



PHD

Studies of viruses and virus-like agents infecting woody ornamentals

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STUDIES OF VIRUSES AND VIRUS-LIKE AGENTS
INFECTING WOODY ORNAMENTALS.

Submitted by Nualphan Ngamyeesoon,
B.Sc., M.Sc. for the degree of Ph.D
of the University of Bath
1989

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SUMMARY

A survey of trees and shrubs for virus infection found 24 species of woody ornamentals with virus-like symptoms. Nine isolates were obtained by sap transmission to herbaceous indicator plants. Viruses isolated from *Cassia corymbosa* and *Caryopteris clandonensis*, and to a lesser extent *Senecio greyi*, *Forsythia intermedia* and *Spiraea bumalda*, formed the basis of the present study. The viruses obtained represented at least three distinct taxonomic groups.

Cucumber mosaic virus (CMV) was obtained from *Caryopteris clandonensis* (one isolate) with mild chlorotic symptoms, and from *Senecio greyi* (two isolates) associated with either mild chlorotic symptoms (SV1) or reduced growth (SV2). The isolates were identified serologically and by analysis of double-stranded RNA from infected plants. The isolation of CMV was apparently a first record in the UK for these species.

A virus with long flexuous particles was isolated from the shrub *Cassia corymbosa* with severe mosaic-type symptoms, the first record in this species. Leaf squash preparations showed a range of particle sizes in the electron microscope,

28% of which were over 1000 nm in length. The virus was tentatively identified as a closterovirus, on the basis of particle size and fragility and analysis of dsRNA. The virus was not transmitted by *Myzus persicae*, but a single lesion isolate was returned to *Cassia corymbosa* seedlings which showed typical symptoms of disease.

Members of the nepovirus group were isolated from three shrubs: strawberry latent ringspot virus (SLRV) from *C. clandonensis* with stunting and reduced leaf size, a first record in this genus; arabis mosaic virus from *C. corymbosa* and *Spiraea bunalda* with either an apparently latent infection or vein yellowing, respectively; and raspberry ringspot virus from *Forsythia intermedia* with chlorotic rings and lines. Isolates were identified from serological reactions in agar gel double-diffusion, and an antiserum was prepared to SLRV.

Enzyme-linked immunosorbent assay (ELISA) was used to detect CMV in herbaceous and woody hosts, and to detect SLRV in *C. clandonensis* explants grown in vitro. Analysis of virus-specific dsRNAs was used to detect and identify viruses in herbaceous and woody hosts.

Thermotherapy or chemotherapy, in combination with tissue culture, reduced levels of SLRV in *C. clandonensis* explants below levels detectable by infectivity assay or ELISA.

ABBREVIATIONS

a. Standard abbreviations are not included here:

Abs or A	= absorbance (at stated wavelength)
BA	= benzyladenine
c.	= approximately
CARNA 5	= CMV-associated RNA 5
cm ²	= square centimetres
CPG	= controlled pore glass
cv.	= cultivar
DEP	= dilution end-point
DIECA	= sodium diethyldithiocarbamate
DNase	= deoxyribonuclease
dsRNA	= double-stranded RNA
EDTA	= ethylene diamine tetraacetate
ELISA	= enzyme-linked immunosorbent assay
HCP	= hydrated calcium phosphate
LIV	= longevity <u>in vitro</u>
Mr	= relative molecular mass
PBS	= phosphate buffer saline
PEG	= polyethylene glycol
PVP	= polyvinyl pyrrolidone
RNase	= ribonuclease
SDS	= sodium dodecyl sulphate
ssRNA	= single-stranded RNA
syn.	= synonym
TIP	= thermal inactivation point

U.V. = ultraviolet
var. = varieties (botanical variety)
v or vol. = volume
w. = weight

b. Viruses

ArMV = arabis mosaic
CLRV = cherry leaf roll
CMV = cucumber mosaic
RRV = raspberry ringspot
SLRV = strawberry latent ringspot
TBRV = tomato black ring
TMV = tobacco mosaic
TNV = tobacco necrosis
TobrV = tobacco ringspot
TomRV = tomato ringspot

CHAPTER 1 : GENERAL INTRODUCTION.

Virus diseases of plants have been recognized since 1892, when Iwanoski demonstrated that the infectious agent of tobacco mosaic virus (TMV) would pass through a bacteria-proof filter. But it was not until the 1930's when the electron microscope was invented that viruses could be "visualised". However, symptoms of virus-like diseases on ornamentals have been known for many years. The earliest observations dated from the sixteenth and seventeenth centuries when tulips with "broken" or variegated flowers, featured in the works of the Rembrandt School of Dutch flower Painters. By 1643, growers had found that this flower character could be transmitted to healthy plants by grafting and there was a suggestion that the variegation was due to a disease as early as 1670 (cited in Smith, 1977; and Bos, 1983).

There are a number of other early records concerning graft transmission of viruses between ornamentals. Lawrence (1715), Blair (1719) and Cane (1720), for example, all referred to the transmission of the yellow variegation of jasmine foliage. Although many later workers considered that such "infectious variegation" of trees and shrubs was due to disease, this view was not widely

accepted, and some workers thought the transmission might concern the influence of scion on stock rather than disease (Atanasoff, 1935). In 1869, Morren demonstrated that the agent responsible for mosaic symptom of *Abutilon striatum* Dickson var. *thompsonii* (syn. var. *spurium*) could be passed from a scion leaf to a green stock by leaf petiole insertion beneath the bark of the stock. The leaf often died, indicating that mosaic on the stock could not be due to a sustained influence of scion on stock, but to transmission of a disease-causing agent. However, the mosaic disease of *Abutilon* was not sap transmissible (Baur, 1904).

The first reports of virus infection of woody ornamentals were those of Atanasoff (1935) and Brierley (1944). Many of the reports, however, referred to uncharacterise graft-transmissible agents. Cooper (1979) reviewed infection of a range of trees and shrubs hardy in the U.K., excluding fruit crops, which were discussed by Posnette and Cropley (1963), and small fruits and vines, which were discussed by Frazier et al. (1970).

Atanasoff (1935) referred to 31 genera in 22 families, suspected of being infected with viruses or virus-like agents, while Cooper (1979) had extended this list to 86 genera in 49

families. He included viruses, rickettsia and mycoplasma-like organisms, and other graft-transmissible agents, in addition to well-characterised viruses.

Many of the early studies of virus diseases of trees and shrubs were primarily concerned with infection of economic crops. The number of identified viruses causing diseases of trees or shrubs, however, is markedly less than of those causing diseases to food and fibre crops. This may reflect a greater degree of resistance in tree species, or a lack of pronounced symptoms whenever a tree species is infected with a virus. The pressure for greater productivity of food and fibre crops has limited the resources available for tree production, but there has been an increasing awareness, in the last decade, of the importance of viruses affecting trees and shrubs (e.g. Cooper, 1979; Perkins, 1987; Webster, 1988).

The first major study of the viruses of a wide range of native and amenity woody plants was that of Schmelzer in East Germany in 1971. He found that cucumber mosaic virus (CMV) and the nematode-borne polyhedral viruses (nepoviruses; sensu Cadman, 1963), arabis mosaic (ArMV) and tomato black ring viruses (TBRV), were relatively common in

woody species. More recently Millikan (1982) reviewed virus diseases of shade and ornamental trees, and reported that of 10 viruses identified as causing disease in 16 different tree species, over half were nepoviruses. The importance of nepoviruses and CMV in trees and shrubs was confirmed by Cooper (1979). Out of 43 host families surveyed 26 were affected by nematode-borne viruses while 17 were affected by CMV and to a lesser extent by alfalfa mosaic virus both of which are spread by aphids.

The nepoviruses naturally infect a wide range of endemic wild plants, including weeds, and particularly perennials, which provide the more stable habitat preferred by the nematode vectors (Thresh, 1980). Viruses such as ArMV, cherry leafroll virus (CLRV) and strawberry latent ringspot virus (SLRV) are frequently found in hedgerow plants (e.g. Harrison and Winslow, 1961) which are often symptomlessly infected. The most characteristic symptom induced by nepoviruses is ringspot as reported for CLRV in *Sambucus racenosa* (Schmelzer, 1966) and raspberry cultivars with RRV (Murant, 1981 a). Other common types of symptom include the leaf flecking and leaf mottling seen in raspberry and strawberry infected with SLRV,

raspberry ringspot virus (RRV) and ArMV (Murant, 1981a). In roses, leaf flecking and mottling were often accompanied by a leathery texture to the leaves (Ikin and Frost, 1976). These latter authors also reported an association in rose cultivars between the death of scion buds and infection of the rootstock (*Rosa rugosa*) with SLRV. Accurate diagnosis of nepoviruses from field symptoms alone is difficult, however, because of the wide range of diseases caused by individual viruses and the variety of response given by different cultivars of the same species.

Many nepoviruses are transmitted through the seed of plants often to more than 50% of the progeny (Lister and Murant, 1967), which may then act as a reservoirs for subsequent spread by previously non-infective nematodes, or nematodes rendered non-infective after a period of overwintering fallow (Murant and Lister, 1967). Seed-borne virus may also be important in commercial seed lots of some crops (Hicks et al., 1986), but infected seed may generally be undetected because infected seedlings are often symptomless (Murant, 1981 b).

The vectors of nepoviruses are ectoparasitic dorylaimid nematodes of the genera

Longidorus and *Xiphinema* (Harrison, 1977). Members of both genera are essentially polyphagous, and the apparent association with perennial vegetation, such as woodland and grassland, may be due to their sensitivity to disturbance (Taylor and Brown, 1976; Murant, 1981 b). However, viruses such as RRV and TBRV, with *Longidorus* vectors, are found less frequently in woody hosts than ArMV, TomRSV and TobRSV. This may be because longidorids tend to be associated with shallow rooted herbaceous crops rather than with tree roots (Lamberti et al., 1975).

Nematodes are responsible mainly for local spread of nepoviruses because of their slow migration through the soil (Harrison and Winslow, 1961; Taylor and Thomas, 1968). Long distance spread presumably take place by dispersal of infected seed or pollen (Harrison, 1977; Murant, 1981 b), or by the transfer of nematodes with soil on plant material.

Apart from the nepoviruses, cucumber mosaic virus (CMV) is another agent found to infect woody ornamental plants. Most isolates of CMV have a wide host range and polyphagous mobile aphid vectors, with little virus-vector specificity (Kaper and Waterworth, 1981). These properties probably contribute to the

prevalence of CMV in both crop plant and weed species.

Several woody plants are reported to be natural hosts for cucumber mosaic virus including *Jasminum nudiflorum* (Winter jasmine) (Waterworth 1971) and *Viburnum* (Schmelzer, 1970).

The most common symptom induced by CMV in plant hosts is a leaf mosaic (e.g. McClean et al., 1962; Brunt, 1966; Demski, 1969; Gracia and Feldman, 1977). The severity of disease, however, may range from no obvious symptoms in some crops to plant death (Marrou and Putz, 1973). Some isolates of CMV induce other symptoms such as ringspot (Paludan, 1970) which may be confused with those of nepoviruses, and one strain causes fruit woodiness (Taylor and Kimble, 1963).

Cucumber mosaic virus is transmitted in a non-persistent manner by a large number of aphid species, and can be acquired with probes of one minute or less. Furthermore, there is no latent period before the virus can be transmitted to new plants. However, aphids retain the virus for less than four hours, and it is not transmitted to progeny aphids (Kaper and Waterworth, 1981). In view of these properties it may be surprising that the

virus is not even more widespread in woody hosts. Furthermore, Tremaine (1977) did not observe any natural spread of CMV from infected peach seedlings to adjoining peach or sour cherry in ten years of observations.

There are reports of transmission of CMV by seed (Phatak, 1974) particularly in weeds, and rates as high as 95% have been recorded in *Echinocystis* (Neergard, 1977). As with other viruses of woody ornamentals CMV can also be disseminated via routine vegetative propagation procedures.

Many viruses infecting trees and shrubs are widely distributed, for example, CMV is found throughout temperate regions of the world (Francki et al., 1979). Among the nepoviruses, however, members have a more restricted distribution, presumably determined by that of their natural vectors (Harrison and Murant, 1977). The group is represented in Europe by viruses such as ArMV, RRV, SLRV and TBRV, and in North America, by tobacco ring spot virus (TobRV) and tomato ringspot virus (TomRV) (Murant, 1981 a).

A literature survey showing the distribution of nepoviruses and CMV in common genera of woody ornamental trees and shrubs is shown in table 1

Table 1 Genera of woody ornamentals as natural hosts
for nepoviruses or cucumber mosaic virus

Host	Viruses	Investigators
<i>Abeliophyllum sp.</i>	TBRV	Schmelzer (1974)
<i>Aesculus sp.</i>	SLRV	Schmelzer & Schmidt (1968)
<i>Aristolochia sp.</i>	CMV	Lihnell (1951)
	ArMV	Schmelzer & Schmidt (1968)
<i>Berberis sp.</i>	CMV	Wilkinson (1953)
<i>Betula sp.</i>	CLRV	Schmelzer (1972)
<i>Buddleia sp.</i>	CMV	Smith (1952)
	SLRV	Van Hoof & Carron (1975)
<i>Buxus sp.</i>	ArMV	Lister in Frazier <u>et al.</u> (1970)
<i>Caryopteris sp.</i>	TBRV	Schmelzer (1968)
	CMV	Schimanski <u>et al.</u> (1977)
	ArMV	Schimanski <u>et al.</u> (1977)

<i>Celastrus sp.</i>	TBRV	Schmelzer (1974)
<i>Chionanthus sp.</i>	TBRV	Schmelzer (1970)
	CMV	Schmelzer (1970)
<i>Clematis sp.</i>	CMV	Thomas (1975)
<i>Cornus sp.</i>	CLRV	Waterworth & Povish (1972)
	ArMV	Barnett & Baxter (1976)
	TobRSV	Reddick <u>et al.</u> (1977)
	TomRSV	Reddick <u>et al.</u> (1977)
	CMV	Barnett & Baxter(1973)
<i>Daphne sp.</i>	CMV	Boning (1963)
	ArMV	Lister in Frazier <u>et al.</u> (1970)
<i>Escallonia sp.</i>	CMV	Thomas (1975)
<i>Euonymus sp.</i>	SLRV	Baur (1908)
	CMV	Barnett & Baxter (1973)
<i>Forestiera sp.</i>	TBRV	Schmelzer (1974)
<i>Forsythia sp.</i>	ArMV	Schmelzer (1962-63)

	TobRSV	Tiangco & Varney (1970)
	RRV	Tiangco & Varney (1970)
	TomRSV	Schmelzer(1957) (1962-63)(1974)
<i>Fraxinus sp.</i>	ArMV	Cooper (1975)
	TobRSV	Hibben & Bozarth (1972)
<i>Hedera sp.</i>	ArMv	Cooper & Sweet (1976)
	SLRV	Cooper & Sweet (1976)
<i>Hydrangea sp.</i>	TomRSV	Brierley (1954)
	TobRSV	Anderson (1958)
	TomRSV+ArMV	Schmelzer (1970)
	CMV	Tamura & Komuro (1967)
<i>Ilex sp.</i>	TobRSV	Waterworth & Povisk (1977)
<i>Jasminum sp.</i>	CMV	Waterworth (1971)
	ArMV	Cane (1720)
	TobRSV	Morton <u>et al.</u> (1977)

<i>Juglans</i> sp.	CLRV	Savino <u>et al.</u> (1977)
<i>Laburnum</i> sp.	ArMV	Cooper & Sweet (1976)
<i>Leycesteria</i> sp.	CMV	Hollings (1961 a, b)
<i>Ligustrum</i> sp.	CMV	Smith (1957)
	ArMV	Jankulova & Schmelzer (1974)
	TomRSV	Novak (1969)
	CLRV	Schmelzer <u>et al.</u> (1969) cited in Schmelzer (1972)
<i>Lonicera</i> sp.	CMV	Lihnell (1951)
<i>Lycium</i> sp.	CMV	Schmelzer & Schmidt (1968)
<i>Magnolia</i> sp.	CMV	Schmelzer & Schmelzer (1968)
<i>Nandina</i> sp.	CMV	Brierley & Smith (1960)
<i>Passiflora</i> sp.	CMV	Taylor & Greber (1973)
<i>Paulownia</i> sp.	CMV	Schmelzer (1969)
<i>Prunus</i> sp.	CLRV	Schimanski <u>et al.</u>

		(1975)
<i>Ptelea sp.</i>	ArMV	Schmelzer (1962-63)
	CLRV	Schmelzer (1972)
<i>Rhamnus sp.</i>	CMV	Quiaoit & Fulton (1966)
<i>Robinia sp.</i>	SLRV	Schmelzer (1969)
<i>Rosa sp.</i>	ArMV	Ikin & Frost (1974)
	TomRSV	Halliwel & Milbrath (1962)
	TobRSV	McDaniel <u>et al.</u> (1971)
<i>Romneya sp.</i>	CMV	Hollings (1961)
<i>Sambucus sp.</i>	ArMV	Cadman (1960)
	TomRSV	Schmelzer (1966)
	CLRV	Schmelzer (1966)
	SLRV	Lister (1964)
	CMV	Uyemoto & Gilmer (1971)
<i>Spiraea sp.</i>	ArMV	Schmelzer (1970)
<i>Staphylea sp.</i>	ArMV	Schmelzer (1974)
<i>Syringa sp.</i>	SLRV	Van der Meer(1976)
	ArMV+CLRV	Novak & Lanzova (1975;1977)

	ArMV+TomRSV	Schmelzer (1970)
<i>Ulnus</i> sp.	CLRV	Swingle et al. (1943)
<i>Viburnum</i> sp.	CMV	Schmelzer (1970)

Although the more extensive studies pointed to the prevalence of CMV and the nepoviruses among trees and shrubs, a number of other viruses have also been reported. Thus tobacco rattle virus, another nematode-borne virus (with elongate particles), has been reported from *Betula* (Cadman cited in Schmelzer, 1972), *Chioanthus* (Schmelzer, 1970) *Forsythia* (Schmelzer, 1957, 1974) *Hydrangea* (Schmelzer, 1970) and *Syringa* (Huttinga, 1972). The fungal-borne tobacco necrosis virus was isolated from the roots of *Betula*, conifer, *Populus* and the leaves of *Kuonynus*, *Hydrangea*, and *Sambucus* (Cooper, 1979). Other than CMV the most commonly found virus with an airborne vector that infect trees and shrubs is alfalfa mosaic. This virus has been recorded from *Caryopteris*, *Cistus*, *Forsythia*, *Hebe*, *Hydrangea*, *Lavandula*, *Rhannus*, (Cooper, 1979) *Buddleia* (Perkins and Hicks, 1989) and *Viburnum* (Cooper, 1979). Several other viruses such as those in the ilar group are also found quite

widely in trees and shrubs (Cooper, 1979) but generally in rosaceous species grown for fruit. They also naturally infect flowering cherries (Colin and Verhoyen, 1975) and roses (Ikin and Frost, 1976) as well as *Aesculus* spp. (Sweet and Barbara, 1979), *Betula* spp., *Chaenomeles loagenaria* and several other woody ornamental genera (Sweet and Campbell, 1976; Cooper, 1981).

Some ilar viruses are seed and pollen borne (Fulton 1966), but no other means of natural transmission is known, and their prevalence in fruiting and ornamental stock is mainly attributable to vegetative propagation of infected plants (Hicks, 1979). It is suprising that some common viruses with wide host ranges, like tobacco mosaic virus, are not found more frequently in woody species. Low virus concentrations and high concentrations of inhibitors in sap extracts may have contributed to the low frequency of detection. Thus, a tobamovirus from rose could only be isolated using partially-purified extracts from petals (Hicks and Frost, 1984) and could not be isolated from leaves.

Some viruses appear to have a relatively limited distribution; for example, daphne virus S apparently only infects members of

the genus *Daphne* (Forster and Milne, 1975, 1978). This is possibly due to a genuine host-specificity. Some viruses, however, are apparently naturally host-restricted, whilst having a wide experimental host range; for example, *Lonicera* latent virus is only found naturally among certain members of the genus *Lonicera*, but is mechanically transmissible to several *Chenopodium* and *Nicotiana* spp. (Brunt and Vander Meer, 1984).

From a commercial point of view most virus infections of woody plants are undesirable and overall cause considerable economic losses to the industry. Problems arise particularly in woody species because these are frequently propagated vegetatively (buds, grafts, cuttings) and propagules taken from a plant systemically infected with virus will often, though not always (e.g. Pacumbaba, 1985), contain virus. Losses in yield and vigour have been reported by many authors. For example, Sweet (1978, 1979) demonstrated that graft inoculation of *Fraxinus americana* L. trees with ArMV made the trees shorter and had a smaller growth than healthy controls. Similarly, Thomas (1981a/b) showed that grafted rose bushes artificially infected with PNRSV had fewer, thinner, shorter shoots than healthy plants. In addition, infected

plants produced smaller blooms and flowered late. Bushes graft-inoculated with ArMV or SLRV gave similar results (Thomas, 1982).

A problem often arises because many woody plants infected with viruses, such as the nepoviruses, or CMV, do not show clear-cut disease symptoms, or if they do symptoms develop sporadically (Fulton, 1966, Hicks, 1979). Even plants infected by so-called latent viruses, however, may show significant losses of yield and performance (Converse and Tanne, 1984). Such plants are difficult to remove from the propagation system, as they have been selected over the years for their apparent vigour, and are likely to be 'tolerant' of infection. The loss in yield and quality associated with infected plants often results in considerable financial loss. These losses can be both direct, where plants show obvious symptoms, and have to be discarded, or indirect, which, are costs involved in the maintenance of diseased plants which are subsequently unmarketable (Bos, 1983).

The presence of virus in a tree or shrub may also prevent the export of plants to countries with strict quarantine regulations. To avoid this problem, many countries have introduced schemes for the production of virus-free material.

In the UK certification schemes for the production of pathogen-free stock have been available for many years for several crops including strawberries, potatoes and fruit trees. These schemes have been discussed by Webster (1988) for north European countries.

In the U.K. a "Clonal Selection Scheme" for woody ornamentals was started at Long Ashton Research Station, Bristol in 1976, but was transferred to East Malling Research Station in 1983. The long term objectives of the scheme were to identify superior clones of important species or cultivars and make them available to the hardy nursery stock industry for general distribution.

The operation of the "Clonal Selection Scheme" in the U.K. is presented in Fig 1. (after Webster, 1988)

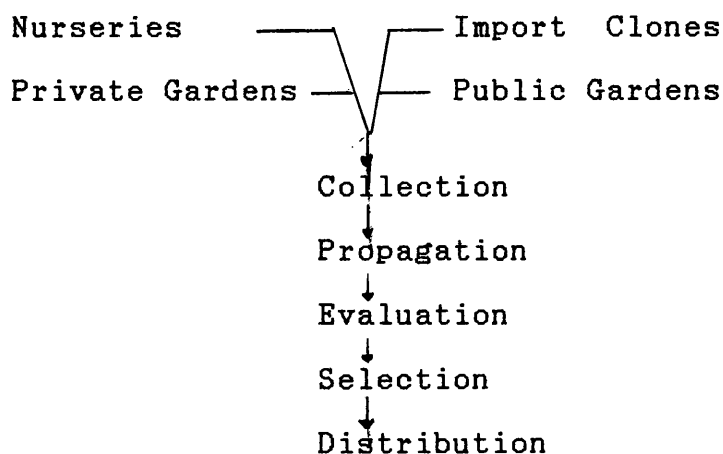


Figure 1 Flow chart of Clonal Selection Scheme

Assessment of clones for resistance to or freedom from viruses forms an important part of the scheme shown in Fig 1. If species or cultivars with desirable characters are found to be infected with virus and it is economically justified, then the application of methods for virus elimination will need to be considered. Various control measures have been developed in order to clean up existing infected material including thermotherapy, and meristem-tip culture often used in combination with thermotherapy (Walkey, 1980). Although use of antiviral chemicals on a field scale is not commercially viable or desirable at the present some success has been reported for a combination of chemotherapy and tissue culture (Cassells, 1987).

Thermotherapy

High temperature treatment has been widely used in the production of virus-free plants and has been reviewed by Nyland and Goheen (1969). The treatment usually involves an immersion of dormant plant material in hot water (c.30°-54°C.) for periods of several minutes or hours and growth of plants in hot air (35°-40°C.) for periods of weeks or months. Hot water treatment has been commonly used for controlling virus and virus-like

diseases of sugar cane (Hollings, 1965). Among woody plants, this method has been used occasionally; for example, Kunkel (1936) eliminated several viruses from peach trees by immersion in water at 50°C. for 10 min. Nyland (1959) inactivated cherry necrotic rusty mottle virus in sweet cherry budsticks by treatment at 50°-52°C. for 5 -15 min. The most generally used method, however, is hot air treatment and a number of woody ornamentals have been freed of viruses by this method, in most cases with significant improvement in vigour. Thus, Humphrey et al. (1973) reported that when *Jasminum magnificum* Lingelsh plants showing a chlorotic mottle were grown at 38 °C for 19 weeks they produced symptomless shoots and yielded cuttings which grew into more vigorous plants, although they reported no indexing for virus.

Chemotherapy

The use of chemicals to suppress virus symptoms and multiplication in infected plants has recently been reviewed by Tomlinson (1982). In general, the evidence suggested that although many chemicals are able to suppress virus symptoms or multiplication, few, if any, eliminated virus completely. In addition, many proven antiviral compounds have been unsuitable for use on crops

because of their cost and phytotoxicity.

Recent chemotherapeutic studies suggested that a more promising approach may be the incorporation of antimetabolite chemicals such as ribavirin (syn. Virazole) into tissue culture media (Shepard, 1977; Cassells and Long, 1980). This chemical blocks virus replication in infected tissue and, presumably, while virus synthesis is stopped, degradation of existing virus continues until eradication has occurred (Simpkin et al. 1981).

The antiviral chemical adenine arabinoside (syn. Vira A or Vidarabine) has also been used in culture medium to eradicate two unidentified viruses from sweet potato (Stone et al., 1978) and a complex of viruses from *Ullucus tuberosum* (Stone, 1982).

Meristem-tip culture

Meristem-tip culture has frequently been used to obtain herbaceous plants free of viruses, but has been exploited less often for woody species (Wang and Hu, 1980) despite the potential for in vitro culture of such plants (Pierik, 1975; Abbott, 1977; Vasil and Vasil, 1980). For example, Jones and Vine (1968) eliminated gooseberry vein-banding virus from gooseberry by meristem culture.

Among ornamentals, Cohen and Lagal (1976) have reported the elimination of daphne virus S from several *Daphne spp.* and Duran and Morand (1978) obtained *Buddleia davidii* plants free of CMV using this technique.

In some cases, however, it has proved difficult to eliminate viruses by heat therapy or meristem culture alone, but a combination of the two techniques has proved successful, for instance, Sweet et al., (1979) used heat therapy at 37°C. for 4 weeks, followed by meristem excision to eliminate viruses from *Daphne spp.*

In virus disease control, distinguishing between research diagnosis and routine indexing is important (Lawson, 1981). Research diagnosis is used to determine if virus is present and then to identify it. This often involves laboratory research in virus purification, characterisation, and strain differentiation in a small number of samples. Routine indexing is the detection of known viruses and usually involves testing a large number of samples. The choice of indexing procedure may depend on the type of ornamental crop and the stage of growth when the test is performed. Bioassay, serology and electron microscopy have been used for virus indexing.

Sap inoculation (transmission) is the primary method used in routine indexing for several of the economically important viruses in ornamentals. This involves inoculation of buffered sap extracts to one or more indicator plants sensitive to the virus under test. Symptoms on test plants may develop within a few days or weeks and indicate the presence of virus in the original host. The choice of a particular test procedure, however, is determined by both the virus to be detected and the host to be sampled. One host may have inhibitors that prevent reliable transmission without the use of chemical additives while the same virus may be more readily transmitted from another host. Other factors that influence bioassays include the effects of light and temperature on symptom expression and virus content of the naturally infected host and on the susceptibility of the test plant used in the bioassay. Uneven distribution of the virus in vivo can also make the test unreliable (Converse, 1978; Hamdorf, 1982).

One of the most important diseases of ornamentals chrysanthemum stunt, is still detected commercially by graft indexing. A method using polyacrylamide gel electrophoresis however, can detect the stunt agent in as little as 50 mg of

infected tissue (Horst and Kawamoto, 1980).

Serological assays for virus detection are based on the reaction between viral antigens and specific antibodies. Serological tests are often used to confirm visual diagnosis or the results of mechanical transmission tests. Recently, Voller et al ., (1976) and Clark and Adams (1977) introduced enzyme-linked immunosorbent assay (ELISA) to detect plant viruses.

The use of ELISA has been rapidly expanded for the detection of viruses in routine indexing programmes, where it combines high sensitivity with low cost, and is readily suited for the processing of multiple samples. Symons (1983) a minimum of 20 million ELISA assays are done worldwide each year to diagnose plant viral infections.

Electron microscopy is a method commonly used to identify a virus on the basis of its size and shape. Virus particles can be observed in crude sap extracts from some infected species. A recent advance combines serology and electron microscopy using the technique of immune electron microscopy (Milne and Luisoni, 1977). The method was slightly more sensitive than the bioassay procedure

for detecting viruses and was comparable to ELISA.

In the present study, the objectives were to continue the earlier studies of Perkins (1987) and investigate the presence of viruses and virus-like agents in woody ornamental material being assessed at Long Ashton under the Clonal Selection Scheme. In addition, woody ornamental shrubs grown at the University of Bath or plants grown locally in parks and gardens in the Avon / Wiltshire area and occasionally further afield were also surveyed for virus infection. Where virus was isolated attempts were made to characterise and identify it and, in some cases, where purification was achieved, to produce a specific antiserum. A further aim for some woody ornamentals was to initiate and establish infected material in vitro and to investigate procedures for the elimination of virus from infected plants, with emphasis on tissue culture. Where virus-free material of particular species was available, re-inoculation experiments were done to demonstrate pathogenicity of single lesion isolates and to satisfy Koch's Postulates.

CHAPTER 2 : MATERIAL AND METHOD

2.01 Source of infected trees and shrubs

Infected material came from trees or shrubs planted at Long Ashton research Station under the CSS, woody ornamental shrubs grown at the University of Bath or locally in parks or gardens in the Bath / Wiltshire area. One source of *Senecio greyi* came from a cutting of a plant grown in a park in Norfolk. Most of the shrubs tested were associated with virus-like symptoms at some time, although not all showed symptoms at the time of sampling, which was normally summer during periods of active growth. Some infected shrubs were transplanted from their original site and grown in buckets of containing sterilised John Innes compost.

2.02 Test plants

2.02 a)Seed sources

Seeds of the following plants were obtained from the Department of Plant Biology at the University of Bath, unless otherwise stated.

Amaranthaceae : *Gonphrena globosa* L.
(globe amaranth).
Suttons Seeds Ltd.

- Chenopodiaceae : *Chenopodium album* L.
(fat hen), Long Ashton
Research Station, Bristol.
C. amaranticolor
Costa and Reyn.As above
C. foetidum Schrad.
C. murale L.As above
C. quinoa Willd.,
- Cucurbitaceae : *Cucumis sativus* L.
(cucumber) "Parisienne
Pickling" and "Marketer"
Sharpes and Co.PLC.
- Leguminosae : *Phaseolus vulgaris* L.
(French bean) "The Prince"
Sharpes and Co.PLC.
Vicia faba L.(broad bean)
Sutton and Son Ltd, Reading.
Pisum sativum (pea)
"Montieth" and "Onward"
Vigna sinensis L.(cowpea)
"Itta", "Bushita Max" and
"M.I. 35", Seed Certification
Centre, Sri Lanka.
Vigna sesquipedalis
(long yard bean)
Glycine max "PM 78-13"
and "Bossier"

Vigna radiata type 51 and
 "H.I.4"
Cassia corymbosa Lam.
 Leam Parks Dept, Monaco.
C. obtusifolia
C. occidentalis
C. tora
Trifolium hybridum
 "Alsika clover"
T. repens "White clover"
 Cruciferae : *Brassica pekinensis*.
 Solanaceae : *Datura stramonium* L.
 "tatula"
Lycopersicon esculentum Mill.
 (tomato) "Moneymaker",
 Samule Yates Seeds Ltd.
Nicotiana clevelandii Gray.
N. debneyi Domin.
N. glutinosa L.
N. megalosiphon
 Huerck and Muell.
N. rustica L.
N. sylvestris
 Speg and Comes
N. tabacum L.(tobacco)
 "White Burley"(sel.
 Judy's Pride) and "Xanthi"

Umbelliferae : *Apium graveolens* L.
(celery) "Dulce Pers"
Daucus carota L.(carrot)
"Chantenay", Red.Cored,
Suttons Seeds Ltd.
Verbenaceae : *Caryopteris clandonensis*
University of Bath

2.02 b) Culture of herbaceous test plants

Certain seeds were given a pre-or post-sowing treatment to improve germination. All *Cassia spp.* seeds have thick seed coat, and untreated seeds seldom germinates. Therefore, the seeds were scarified at the chalazal end using sand paper, then soaked in water for 2-3 hr before sowing in 8.5 x 6 inch tray with Levington seedling compost. The trays were covered with a piece of glass to maintain humidity, put on a sand bottom-heated bench in the mist house. Shortly after the primary leaves emerged, seedlings were pricked out, one plant to each pot containing Levington potting compost. They were then transferred to glasshouse conditions as described below.

Seeds of *Nicotiana clevelandii* were sprayed with gibberellic acid solution (100ppm.)

to stimulate even germination, after sowing in Levington potting compost.

Most seeds except those of cucumber and all legume seeds were sown in trays of Universal compost or Levington Seedling compost (Fisons Ltd.) and kept at 20°C. After germination seedlings were placed under daylight/warm white fluorescent tube lighting, giving a uniform illumination of 8,000 lux for 18 hr. a day. The following week, seedlings were pricked out into 9 cm. square pots (plantpax, Essex) of Levington potting compost and transferred to a bench under netting in a glasshouse under 400 W low pressure mercury vapour lamps (Thorn), 1 m. above the benching, extending the photoperiod to 16 hr. The temperature was 15°-35°C.

During summer months the glasshouse was shaded with Coolglass whitewash (PBI.Ltd) to reduce direct incident sunlight and to allow a more efficient control of temperature.

For cucumber, seeds were sown in 8.5 x 6 inch tray with 4 rows each of 5 seeds. The trays were put in the mist house until germination when they were moved to a glasshouse with suitable conditions for plant growth. Bean seeds were also sown separately in 6 inch diameter

pots with 4 seeds in each and transferred to the glasshouse after germination under mist.

2.02 c) Disorders observed on test plants

Occasionally, certain "healthy" test plants showed virus-like symptoms. Both *Chenopodium amaranticolor* and *C. quinoa* seedlings sometimes produce stunted plants with malformed or chlorotic or small white necrotic spots.

However, there was no evidence of virus after inoculation studies. These species also developed edge necrosis after fumigation of the glass-house with nicotine. Similar symptoms of a necrotic flecking showed on *Nicotiana megalosiphon* after nicotine fumigation.

The older leaves of *Gomphrena globosa* often produced a red-brown spots which were similar to the lesions induced by high light intensity in the glasshouse during summer (Francki, 1967).

Phaseolus vulgaris "The prince" occasionally developed systemic browning, distortion and necrosis accompanied by a bright yellow coloration on their trifoliate leaves. Most

of legumes seedlings sometimes showed vein yellowing on primary leaves and young shoots because of high light intensity.

2.02 d) General hygiene and pest disease control

Various precautions were taken in order to prevent the contamination of the glasshouse with unwanted viruses. Infected plants were kept on separate benches from healthy plants and were screened with Tygan netting (Fothergill and Harvey Ltd.) to avoid transmission of viruses by insect.

Gramoxone 100 (ai. paraquat, ICI PLC) was sprayed to remove weeds in the gravel between and beneath glasshouse benches. Plant debris was removed regularly and destroyed. Moreover, the glasshouse was maintained as a non smoking area to avoid transmission of tobacco mosaic virus from cigarette tobacco.

Polypropylene pots and trays were soaked in 5% formalin solution for several days before being washed in detergent and rinsed, while plantpak pots were discarded.

2.03 Chemicals

Unless stated otherwise, all chemicals used were obtained from British Drug House (B.D.H.Ltd) or Sigma Chemical Co. and were analytical grade.

2.04 pH Measurement

The pH of aqueous solution was measured electrometrically using a PW 9409 digital pH-meter (Phillips) calibrated before each use with standard buffer pH 4.0, 7.0 and 9 as appropriate.

2.05 Spectrophotometry

The ultraviolet absorption spectra of partially purified and purified virus preparations were determined using a Shimadzu U.V. visible recording spectrophotometer. Matched-pair 3 ml or 1.5 ml semi-micro quartz cuvettes were used.

2.06 Preparation and inoculation of test plants

To increase plants susceptibility to virus infection, they were placed in the dark box at 26°C. for 24-48 hr. before inoculation (Bawden and Roberts, 1948).

For routine work, particularly during surveys, inoculum was prepared by grinding leaf material in a chilled pestle and mortar with cold 0.05 M potassium phosphate buffer ($K_2HPO_4 + KH_2PO_4$) pH 7.8 (Yarwood and Fulton, 1967). Inoculum was rubbed on leaves with a finger; when several isolates were involved, different fingers were used to reduce possibility of contamination. Between each inoculation, hand and nails were washed and scrubbed with soap and water. Celite (Hyflo Super Cel, Koch Light Ltd) was added to the inoculum immediately before inoculation but, after homogenising (Yarwood, 1968). To aid the disruption of plant tissue, 100-mesh carborundum was often added to the mortar before grinding.

Additives such as polyvinylpyrrolidone (PVP, Sigma or Polyclar AT) 0.01 M sodium mercaptoacetate, 0.01 M ethylenediamine tetraacetic acid (EDTA), 0.01 M sodium diethyldithiocarbamate (DIECA) were sometimes added in an attempt to minimise the effect of virus antagonists (Fulton, 1964) in plant sap. The presence of virus inhibitors and inactivators, such as tannin and other phenolic compounds, in woody plants sap often reduce virus transmission (Fulton, 1966., Gibbs and Harrison, 1976).

The effects of other inhibitors and mucilage were generally reduced by five- or ten-fold dilution of the inoculum in cold isolation buffer, so that the infectivity of the inoculum was retained. Plants were usually rinsed briefly under a trickling running tap immediately after inoculation, placed in a glasshouse, and covered with damp newspaper overnight to shade them and to reduce the effects of inoculation stress.

2.07 Quantitative experiments

Virus infectivity was assayed by counting the local lesions produced after 10-15 days incubation on *Chenopodium spp.* either on leaves or half-leaves of test plants in 4 x 4 or 6 x 6 Latin square designs (Roberts, 1964). Lesion numbers X were transformed to values Y , according to

$$Y = \log_{10} (X+C), \text{ for } \bar{X} > 10, \text{ and}$$

$Y = \log_{10} 1/2 [X + C + \sqrt{X^2 + 2xC}]$, for $1.5 < \bar{X} < 10$ (Kleczkowski, 1968). Results were tested for significance using student's t-test or analysis of variance (Cochran and Cox, 1957., Parker, 1979).

2.08 Establishment of single-lesion isolates

Viruses to be studied were established as a successive single lesion isolates to eliminate mixtures of viruses or

strains. A single lesion was cut out and each ground in a solid watch-glass with a few drops of phosphate buffer. A pinch of celite was added before inoculation to test plants. The procedure was repeated at least three times before one of the final "pure" cultures was bulked up and maintained in a suitable test plants.

2.09 In vitro properties

For the study of in vitro properties, extracted sap from infected *Chenopodium* or *Nicotiana spp.* was prepared in 0.05 M phosphate buffer pH 7.8 1:2 (w/v) (designated undiluted). Infected leaves were homogenised and filtered through two layers of muslin. Samples were treated as indicated below before adding the same amount of celite and assaying for infectivity.

2.09 a) Dilution end-point (DEP)

This is usually quoted as being between two dilutions : the highest dilution that the sample was still infective and the next highest one. To determine the DEP ten-fold serial dilutions of sap were prepared in cold buffer and each tested on test plants using the 6 x 6 Latin square design.

2.09 b) Longevity in vitro (LIV)

Sap samples were divided into a number of 0.5 ml aliquots in small plastic stoppered tubes. These tubes were stored at room temperature (20°-25°C.) without exposing to direct sunlight or at 4° and -18°C. They were then tested for infectivity at intervals.

2.09 c) Thermal inactivation point (TIP)

The crude extract of sap was pipetted, 0.5 ml into each thin-walled glass tubes and store in crushed ice. Each tube was warmed to the desired temperature. After heating for 10 min. in a thermostatically-controlled water bath, the tube was withdrawn, cooled and the sample assayed on test plants. Unheated juice was also assayed as a control. Samples were usually tested for infectivity at 5 °C. intervals from 40°-90°C.

2.10 Aphid transmission experiments

The aphid species, *Myzus persicae*, was maintained on chinese cabbages with 4-5 leaves present. Stock plants on which aphid cultures were reared, were changed at least once a week, providing fresh young plants on which colonies will thrive. The culture was put under a cage

consisting of a wooden frame built over an enclosed tray. The top and sides were covered with muslin cloth as an aphid proof screen. A regular check for parasitized aphids, like Hymenopterae, was done. At first sight of such parasitization new colonies were started in a clear area and a previous stock plant was discarded.

Transmission experiments were based on Noordam (1973) and Hill (1984). Aphids were routinely transferred by carefully tapping the abdomen with artist's brush after breathing on them. Aphids were starved for 1 hr. before the acquisition access feed. This lasted 5 or 30 min. or 2 days; inoculation periods lasted 1 day with 10 aphids per plants and usually 10 plants per treatment.

2.11 Centrifugation

Low-speed centrifugation was carried out at 4°C. in an MSE High Speed 18 refrigerated centrifuge (Fisons PLC) using the 6 x 250 ml or 8 x 50 ml aluminium fixed - angle rotors. A bench top MSE microcentaur was used for centrifugation of small samples.

High-speed centrifugation was performed at 4°C. in an MSE PrepSpin 75

ultracentrifuge using the 8 x 50 ml or 8 x 25 ml fixed-angle rotors, or the 6 x 38 ml swing-out aluminium rotor.

All figures quoted were the maximum relative centrifugal force in g. calculated from nomograms supplied by the rotor manufacturers.

2.12 Electron microscopy

Specimens were examined in a JEOL 100 CX electron microscope. Crude or purified virus preparations were viewed after negative staining on a carbon-reinforced Formvar or Pioloform coated copper grid of 3.05 mm. diameter and 200 mesh (Hill, 1984).

The techniques for sample preparation were as follows :

2.12 a) Leaf dip (Brandes, 1957)

An infected leaf was cut into small (1-3 mm.) wedge-shaped section and drawn through a drop of stain (1-2% aqueous potassium phosphotungstate (PTA) pH 6.5-9) over a period about 30 sec on a grid. Excess stain was removed from the grid with the edge of a filter paper.

2.12 b) Leaf squash (Walkey and Webb, 1968)

A leaf with symptoms was chopped and squashed in a few drops of negative stain between two microscope slides. A small amount of the stain-sap mixture was drawn up on to the clamped grid, using a fine Pasteur pipette. Excess fluid was carefully removed from the grid using a small piece of filter paper touched to the edge of the grid.

2.12 c) Partially-purified preparations

Partially-purified preparations were diluted to 1:5 or 1:10 (w/v) in negative stain containing 0.01% bacitracin as a wetting agent to improve distribution of stain and virus particles. The mixture was then sprayed on to a carbon-coated grid. The grid was drawn across the drop and allowed to dry. Alternatively, the grid was coated with virus and allowed to almost dry before staining.

The sizes of virus particles were estimated by comparison with measurement of the lattice spacing of negatively stained catalase crystals (Wrigley, 1968).

2.13 Virus purification by permeation chromatography on columns of controlled pore glass

Haller (1965, 1967) and Marcinka (1972) have examined the possibility of using this technique to further purify preparation of a range of viruses. Barton (1977) showed that pore glass chromatography compares favourably with sucrose density gradient centrifugation.

Controlled pore glass (CPG) of 120 to 200 mesh and mean pore size 72.9 nm. (Sigma Ltd.) was cleaned of debris before use. The dry CPG was suspended in buffer and washed several times by decantation. The slurry was then resuspended in buffer containing 1% (w/v) polyethylene glycol (PEG 20,000) and degassed for 15 min using a filter pump. When outgassing had stopped the CPG was washed in buffer alone by several decantations.

The beads of CPG approx. 900 x 15 mm. were slowly poured into a 90 x 1.5 cm. Whatman MS-PC 1500 chromatography column. The supporting clamp stand was vibrated continuously as bead slurry was continuously added and buffer was allowed to elute. Degassed buffer was passed through the column overnight by pumping with an LKB Varioplex peristaltic pump. Column packing was checked by running samples of TMV (3.9×10^6 Mr), Blue Dextran

2000 (Pharmacia Ltd 2 x 10⁸), horse spleen ferritin (560000), bovine catalase (240000), bovine serum albumin (67000), ovalbumin (43000).

Virus samples were applied through a three - way valve and eluted using degassed buffer, pumped on to the column at c. 0.8 ml.min⁻¹cm⁻².

Eluates were monitored at 254 nm. with an LKB Uvicord U.V.monitor connected to an LKB 22100 chart recorder. Fractions of c. 4.2 ml were collected using an LKB Ultrorac 7007 fraction collector. All chromatography was conducted at room temperature, but fractions were placed at 4°C after elution. Prolonged (overnight) chromatography at room temperature was avoided to minimise loss of virus due to inactivation. The fractions were concentrated by ultracentrifugation.

Two-void volumes of 2 M hydrochloric acid were used to sterilize the column, followed by washing with buffer to pH 7.5. Routine recoating of CPG with PEG Mr 20000 to prevent loss of virus by absorption, was effected on the column (Barton, 1977).

2.14 Extraction and analysis of double-stranded RNA (dsRNA)

The presence of relative molecular mass (M_r) of double-stranded ribonucleic acid (dsRNA) in extracts from infected plants can be used to detect and identify RNA viruses. The number and size of dsRNA detected by gel electrophoresis is characteristic of a virus or viral group (Dodds et al ., 1984; Jordan and Dodds, 1985). The technique has an advantage over isolation on to herbaceous test plants in that it is less tissue dependent (Jordan and Dodds, 1985).

A modification of the procedure of Morris et al (1983) based on a method described by Morris and Dodds (1979) was used, but with the buffer and additives of Jordan and Dodds (1985).

Samples of plant tissues were powdered in liquid nitrogen using chilled mortar and pestle. The frozen powder was extracted in 1 vol. of double-strength STE buffer (single-strength = 50 mM tris-HCl, 100 mM NaCl, 1 mM EDTA pH 7.0) containing 10 g/l sodium lauryl sulphate, 1 g/l 2-mercaptoethanol and 10 g/l polyvinylpyrrolidone, 1 vol. of water-saturated phenol and 0.5 vol. of chloroform : pentanol (25:1).

The resulting slurry was shaken for 30-45 min. at room temperature before being centrifuged at 6000 g for 20 min. Absolute ethanol was added to the aqueous supernatant (containing cellular nucleic acids) to a final concentration of 17%.

The dsRNA was purified at room temperature by passage through a column of 2.5 g. CF-11 cellulose (Whatman Ltd.), and suspended in STE buffer containing 17% ethanol, in a 30 ml disposable syringe plugged with a filter paper disc. The column was washed with at least 80 ml STE buffer containing 17% ethanol. After extensive washing the dsRNA was eluted from the column in three aliquots of 5 ml ethanol-free STE buffer. The first 5 ml was discarded as this contained little dsRNA (Morris et al., 1983). The final 10 ml was collected. DsRNA was precipitated by addition of 2.5 vols. of ethanol and a few drops of 3 M sodium acetate, pH 5.5 followed by overnight storage at -20°C.

The precipitate was collected by centrifugation at 8000 g for 20 min. and dried to remove traces of ethanol. Then, the pellet was resuspended in 200 μ l electrophoresis buffer (40 mM tris, 20 mM sodium acetate, 1 mM EDTA pH 7.8) and one drop of glycerol was applied.

Polyacrylamide tube gels were prepared by mixing together the following modified from Heick (1984) :

4 ml	acrylamide-Bis mixture (15% acrylamide-0.375 Bis)
3.33 ml	3 strength of electrophoresis buffer
4.90 ml	distilled water
0.20 ml	of 10 % (w/v) ammonium per sulfate
0.02 ml	TEMED (N,N,N,N,-tetramethyl- enediamine)

The glass tubes were cleaned and rinsed with distilled water. The solution was added immediately after mixing and overlaid with water with a drawn out Pasteur pipette to give a flat surface as polymerisation commenced. The unused gel tubes were kept at 4°C under electrophoresis buffer.

The purified dsRNA was electrophoresed on 6% polyacrylamide gels cast in 75 x 5 mm (i.d.) glass tubes either at 5-6 mA/tube for 3-4 hr. or 2 mA/tube for 16-18 hr. Gels were pre-run for 30 min. at 2.5 mA/gel.

After electrophoresis gels were stained in ethidium bromide 20 ng/ml in

electrophoresis buffer for half an hour and de-stained in distilled water. DsRNA bands were viewed on an ultraviolet transilluminator at 260 nm. The presence of dsRNA was confirmed after electrophoresis by incubating gels in deoxyribonuclease (DNAase) and in "high" and "low" salt ribonuclease, as described by Hicks and Haughton (1986).

Relative molecular mass (Mr)
"standards" used for estimating the size of unknown dsRNAs were tobacco mosaic virus, cucumber mosaic virus and bacteriophage lambda DNA following digestion with Hind III restriction endonuclease (Boehringer Corporation Ltd.). Relative molecular mass were calculated using the graphical method of Bozarth and Harley (1976).

For dsRNA comparisons 0.6% agarose slab gel type I (Sigma Ltd.) was used by melting agarose powder in 1 strength Tris-Borate (TBE) buffer (0.089 M tris-borate, 0.089 M boric acid and 0.002 M EDTA pH 8.0) and, when the gel solution had cooled (c. 50°C.), pouring it into a minigel mould with the comb (8 tracks) clamped in place. When the gel set (1-2 hr.) the comb was carefully removed from the gel.

Samples of about 20 μ l were mixed with loading buffer containing 0.25% bromophenol blue 0.25% xylene glycol and 30% glycerol in distilled water before loading into the slots of submerged gel in TBE buffer.

Electrophoresis was performed for 3 $\frac{1}{2}$ hr. at 20 volts in a minigel mould and gels were stained in ethidium bromide, as mentioned above.

2.15 Serology

2.15 a) Preparation of antisera

Antisera to virus and healthy *N. tabacum* "Xanthi" were prepared by intravenous and / or intramuscular injections of brown rabbits (Sandy lop) with 0.5 to 1 ml of purified samples adjusted to 0.15 M with respect to sodium chloride. Each injection contained from 0.1 to 1 mg/ml of virus.

Intravenous injections were made into the marginal ear vein. Samples for intramuscular injection were first emulsified with an equal volume of Freund's Complete Adjuvant (Difco Labs) before administering as two injections, one in each flank.

Test bleeds of 5 ml were taken from the ear, starting 2 weeks after the first injection, and the harvest bleed was decided following the titre judged to be the optimum. Blood was allowed to clot overnight at room temperature, and the clear serum pipetted off and further clarified by centrifugation for 5 min. at 5000 g. Antisera was mixed with an equal volume of glycerol and stored in universals at -18°C.

2.15 b) Double diffusion test

The Ouchterlony double diffusion test (Crowle, 1973; Noordam, 1973) was applied on agar or agarose gels on glass microscope slides using a Gelman immunodiffusion kit. New slides were cleaned in 70% (v/v) ethanol, air-dried and then quickly dipped into 1% formvar (w/v) in chloroform solution before air-dried and placed in an immuno-frame. The three slides in each half of the frame were sealed with molten agar or agarose before they were coated with 15 ml of molten agar or agarose.

Gel diffusion test media consisted of 0.01 M potassium phosphate buffer pH 7.6 with 0.003 M sodium azide with coating either 0.75 g Ionagar No.2 (Oxoid Ltd.) and 0.15 M sodium chloride, or 0.75 g Agarose A (Pharmacia Ltd).

The gels were air-dried for 15 min. and then placed in a moist chamber for at least 1 hr. before cutting the wells with a Gelman punch. Wells were 3 mm. in diameter and 5 mm. apart; the central well was usually surrounded by six or eight peripheral wells. Antigens and antisera were diluted with 0.01 M phosphate buffer, pH 7.6, containing 0.15 M sodium chloride. Wells were filled using glass micropipettes and slides were incubated for 24 hr at room temperature to encourage the diffusion of reactants, before placing at 4 °C. Precipitates were observed against a dark background using an oblique light source.

For the tests made with unpurified sap from infected and healthy leaves, a few drops of saline solution (0.85% w/v) was added to the sap and centrifuged at slow speed to remove coarse cellular debris. The supernatant was pipetted off and used as undiluted samples.

Healthy sap and normal serum controls were included in all tests.

2.15 c) Enzyme-linked immunosorbent assay (ELISA)

Immunoassays employing enzyme labels were first described independently in 1971 by

Engvall and Perlmann, who termed them enzyme-linked immunosorbent assay (ELISA), and by Van Weeman and Schuurs (Maramorosch and Koprowski, 1984).

ELISA was first applied to plant viruses in 1976 by Voller et al. and by Clark and Adams (1977). This represented a landmark in the detection, diagnosis and assay of viruses. ELISA techniques give greater sensitivity over classical precipitation tests by the use of "Enzyme labelling". The technique combines high sensitivity with low cost. It is widely used and accepted by plant virologists. According to Symons (1983) a minimum of 20 million ELISA assays are applied worldwide each year to diagnose plant viral infections.

The procedures falls into two broad categories; "direct" and "indirect" tests. In direct procedure, the antigen is first absorbed and immobilized on the solid phase and then detected by an enzyme conjugated specific antigen. An enzyme substrate is then added to the reactants resulting in a quantifiable colour change. The antigens may be adsorbed directly to the solid phase (antigen coated direct ELISA) or trapped by specific antibody or antibody fragments previously adsorbed to the solid phase. The latter technique

is referred to as double-antibody sandwich ELISA, DAS (Clark and Adams, 1977). In indirect ELISA, the antibody is not conjugated with an enzyme label; the antibody is added to the antigen which is immobilised to the solid phase and is then itself detected by the addition of an enzyme labelled anti-immunoglobulin.

The main disadvantage of DAS ELISA is that a separate conjugating enzyme must be developed for each antibody, even for closely related strains of a virus. Thus, direct ELISA is antigen specific. Indirect ELISA, however, is more flexible since the conjugate is developed for the anti-immunoglobulin which itself can be used to detect a wide range of antigens. Indirect ELISA can be used, for example, for the quantitative evaluation of strain relationships (Lommel et al., 1982). Furthermore, the use of a protein A enzyme conjugate allows unfractionated antiserum to be used (Mowat and Dawson, 1987).

In the present study, the direct ELISA and double antibody sandwich form of ELISA was done according to Lister's (1979) modification of the original protocol described by Clark and Adams (1977).

The following buffers were used; phosphate-buffered saline (PBS) - 0.01 M phosphate ($\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$) containing 0.15 M sodium chloride and 0.003 M potassium chloride, pH 7.4 ; PBS with 0.5 ml/l Tween 20 (Sigma Ltd.); coating buffer-0.05 M sodium carbonate, pH 9.6; antigen buffer - PBS-Tween containing 20 g/l PVP (Mr 40,000 Sigma Ltd); conjugate buffer-PBS-Tween containing 20 g/l PVP and 2 g/l ovalbumin (Sigma grade V) (PBS-TPO) substrate buffer 97 ml/l diethanolamine adjusted to pH 9.8 with hydrochloric acid.

Gamma-globulin was prepared from 1.0 ml samples of antiserum (dialysed against PBS to remove glycerol) by ammonium sulphate precipitation. The precipitate was resuspended in 2 ml of half strength PBS and dialysed three times against this buffer to remove ammonium sulphate. The preparation was passed through a column of DE 23 cellulose (Whatman Ltd.) housed in a 5 ml plastic syringe, to remove albumins. Gamma-globulin was washed from the column using half-strength PBS, the eluate being monitored at 280 nm and the first protein peak collected. This was concentrated to c. 1 mg/ml (absorbance 1.4) and stored at -18°C .

For conjugation with alkaline phosphatase (Sigma type VII-S), c. 2.5 mg. of enzyme precipitate was collected by centrifugation for 10 min. at 6000 g. and then dissolved in 1 ml of gamma-globulin solution. The mixture was dialysed three times against PBS and 0.6 μ l/ml fresh glutaraldehyde (Sigma, high purity) added. After incubation for 4 hr. at room temperature, glutaraldehyde was removed by dialysis against PBS containing 0.003 M sodium azide. The conjugate was stored at 4 °C. with 5 mg/ml bovine serum albumin (crystallised and lyophilised, Sigma Ltd.).

ELISA was performed using polystyrene microtitre plates (Alpha Laboratories Ltd.). Gamma-globulin was diluted in coating buffer and 200 μ l added to each well using a micropipette. The plate was incubated either for 16 hr. at 4 °C. or 4 hr. at 37°C. in a moist chamber, and then washed 3 times by flooding carefully with PBS-Tween from a wash-bottle.

Test samples were prepared by grinding tissues in antigen buffer and filtering through muslin. Healthy sap and PBS-Tween controls were also prepared. Aliquots of 200 μ l were added to duplicate wells and the plate was incubated as above. An initial wash was discarded immediately to

remove suspended solids, and then the plate was given three standard washes before blotting dry.

Aliquots of 200 μ l of conjugate, diluted in conjugate buffer, were added to each well. Following incubation, the plate was washed as above.

Finally, 200 μ l of freshly prepared p-nitrophenyl phosphate (Sigma Ltd.) at 0.6 mg/ml in substrate buffer was added to each well and incubated for 30 min. at room temperature. The reaction was stopped when necessary by addition of 25 μ l of 3 M sodium hydroxide to each well.

Hydrolysed enzyme substrate was determined by measuring absorbance of 410 nm. (A₄₁₀) using a Titertek Multiskan photometer (Flow Laboratories Ltd.). Readings were taken after the plate had been "blanked" against empty wells. Values of A₄₁₀ greater than twice those of healthy controls were regarded as positive for virus (Thomas, 1980; Hill, 1984).

In some cases, the procedure described by Lommel et al. (1982) was chosen. This procedure can be referred to as "Antigen coated indirect ELISA". It is simpler than "sandwich" procedures in which trapping antibodies are used in

order to immobilise the antigens in the solid phase.

The procedure was as follows; 200 μ l crude extracted sap was added to duplicate or triplicate wells and the plate was incubated at 4 °C overnight. After washing the wells three times as for DAS ELISA, 200 μ l of a specific antiserum diluted in PBS-TPO was added to each well and incubated at 37 °C. for 2 hr. Then, three further washes were given before protein A alkaline phosphatase diluted in PBS-TPO at a desirable ratio was added to each well. Following incubation at 37°C. for 2 hr. the plate was washed. Later, 200 μ l of substrate PNP (p-nitrophenyl phosphate) diluted in substrate buffer at a concentration of 1 mg/ml, was applied to the wells. The plate was covered and incubated at room temperature to allow colour development. The absorbance at 410 nm. was recorded using the automated plate reader.

2.16 Estimation of capsid protein relative molecular mass

The method of Weber and Osborn (1969) was used to study capsid protein relative molecular mass on 7.5 % polyacrylamide gel. The gel was prepared by mixing together the following ingredients (Maizel, 1968).

5	ml	acrylamide-bisacrylamide (30% w/v acrylamide, 0.8% w/v bis- acrylamide)
2	ml	1 M sodium phosphate buffer
0.2	ml	sodium dodecyl sulphate (SDS) (10% w/v)
12.7	ml	distilled water
0.2	ml	ammonium persulphate (10% w/v) fresh
0.02	ml	TEMED (N,N,N',N'-tetramethyl ethylenediamine)

Glass gel tubes (0.6 x 7.5 cm.)(Bio-Rad Laboratories) were soaked in cleaning solution, rinsed extensively and oven-dried before used. Gel was added immediately after mixing, to within 1 cm of the top of each tube, avoiding the formation of air bubbles. Before the gels hardened, a few drops of water were carefully layered on top of the gel solution to give a horizontal meniscus. Gels were stored at room temperature in electrophoresis buffer (100 ml 1 M sodium phosphate buffer and 10 ml of 10% SDS made up to 1 litre in distilled water).

Partially-purified virus samples in 0.01 M sodium phosphate buffer pH 7 containing 1% SDS and 1% 2-mercaptoethanol were dissociated by boiling for 5 min. A few grains of bromophenol blue

and 100 μ l/ml glycerol were added to each protein sample and c. 100 μ l loaded on the gels.

Electrophoresis was performed for 4 hr. at 8 mA/gel, in 0.1 M sodium phosphate buffer containing 1 g/l SDS, using an LKB 3371 DC power supply. Gels were pre-run for 30 min. at 4 mA/gel to remove unreacted persulphate. Glyceraldehyde phosphate dehydrogenase (rabbit muscle Mr 36,000), β -lactoglobulin (Mr 18,400) and serum albumin (bovine Mr 68,000) were applied as a standard marker proteins.

After electrophoresis gel length and distance of dye movement were recorded, and gels were stained for 4 hr. with 2.5 g/l Coomassie blue in 50% (v/v) methanol and 7% (v/v) acetic acid and destained in distilled water. The length of each gel and the position of protein bands were measured. The mobility of each band was calculated with reference to the mobility of the tracking dye (Weber and Osborn, 1969).

2.17 In vitro culture of woody plants

Debergh and Maene (1981) regarded the preparation of stock plants under hygienic conditions as a critical stage in any scheme of plant propagation by tissue culture. Shoot tips

excised from plants grown outdoors were usually contaminated with bacteria and/or fungi when placed on tissue culture media. Therefore, some of the stock plants used in the present study were rooted cuttings grown in the glasshouse for several months before use.

Preparation and subsequent transfers were conducted in a laminar flow cabinet (Centronic Ltd.). Nodal segments (1.5-2 cm. in length) or shoot tips (2 to 5 mm. in length) were surface sterilised for 5 min in 70% (v/v) alcohol then in sodium hypochlorite (20g/l (w/v) available chlorine; Fisons PLC) containing a few drops of Tween-20. Explants were then rinsed in three changes of sterile distilled water and transferred to the sterile medium using flamed forceps (Pink and Walkey, 1984). Any tissue damaged by sterilisation was then excised and discarded, and the remaining explants were cut into 1 cm. length and aseptically placed in either Murashige and Skoog's (MS) medium (Murashige and Skoog, 1962) or woody plant medium containing 0.5 μ M of benzyladenine (BA) (Hisajima, 1981). The medium was adjusted to pH 5.7 with 0.1 N sodium hydroxide. Then it was supplemented with sucrose (20g/l) and agar (lab m M.C.2) at 6 g/l. The medium was heated to dissolve the agar and poured into screw-top jars (c.30 ml/jar) before autoclaving

them for 15 min. at 121°C. and 10^5 N/m².

Cultures were kept at 25°C. (± 1) in a growth room, illuminated with 20 W fluorescent tubes, giving a flux density of 30 μ E/m²/S and 16-hour photoperiod (Dodds and Roberts, 1985). Every 4 weeks, the plants were sub-cultured to fresh medium.

2.18 Cleaning of glassware and apparatus

Glassware and plastic articles which had been in contact with infected plant material or purified virus preparations were immediately washed free of visible contaminants with Detergent Liquid GP (Jeyes Ltd.) or Decon 75 (Decon Labs Ltd.), and soaked overnight. Glassware, ceramic pestles and mortars, and polypropylene centrifuge tubes and bottles were rinsed in tap water and autoclaved for 15 min at 121°C. and 10^5 N/m². Articles were then washed again, rinsed in several changes of distilled water and dried in an oven.

Other non-disposable plastic articles were soaked in detergent and freed of contaminants using an ultrasonic bath, rinsed in distilled water and blotted dry.

Spectrophotometer cuvettes were washed in Decon 75, rinsed in distilled water and stored in 70% ethanol.

The flow-cell of the U.V. monitor was cleaned by passing c. 50 ml of Decon 75 through followed by distilled water.

SECTION A : SURVEY OF WOODY ORNAMENTALS FOR VIRUS INFECTION

To establish isolates for further study, a wide-ranging survey was done for viruses in woody ornamentals. Some of the ornamentals surveyed were part of the clonal selection programme for woody ornamentals organised on behalf of the Industry, by Long Ashton Research Station, Bristol, and, more recently, East Malling Research Station, Kent. Other ornamentals surveyed were grown in park/public gardens or nurseries, mostly in the Avon and Wiltshire areas.

The survey was conducted mainly during the spring and early summer of 1986. This involved, firstly, noting the presence or absence of virus-like symptoms both foliar and effects on growth or vigour, and secondly, taking young leaf samples (with or without symptoms) for virus testing in the laboratory.

To test for virus, leaf tissue was homogenised in a chilled mortar with cold 0.05 M phosphate buffer pH 7.8 containing 75 g/l insoluble polyvinylpyrrolidone (Polyclar AT) (Ikin and Frost, 1976) at a dilution of 1:4 to 1:15 (w/v) depending on the consistency of the inocula. Celite abrasive at a concentration of 4 mg/ml was added

before inoculation of the sap extracts to the following herbaceous test plants : *Cucumis sativus* " Parisienne Pickling " ; *Phaseolus vulgaris* "The Prince"; *Chenopodium quinoa* ; *C. amaranticolor*; *C. foetidum*; *Nicotiana tabacum* " White Burley " (Judy's Pride); *N. clevelandii*; *Brassica pekinensis*. Plants were shaded for 16 hr before inoculation and, after inoculation were rinsed briefly and covered overnight with damp newspaper. Samples from shrubs which had shown symptoms in the field, but were negative in the test, were indexed for a second time using a different buffer composition (0.01 M sodium mercaptoacetate and 0.01 M EDTA in 0.05 M phosphate pH 7.8). In a limited number of cases samples were also assayed for the presence of dsRNA (Hicks et al, 1988). For material grown at Long Ashton, no attempt was made to index all clones of a genus, and in general only plants with virus-like symptoms were selected.

The results are presented in Table 2.

Several virus isolates were obtained during the course of the survey. The symptoms observed ranged from possible mild growth reduction (*Prunus sp.*) to severe stunting of the whole plant (*Caryopteris clandonensis*).

Table 2 Survey of woody ornamental shrubs and trees for virus infection.

Host	L	S	T
<i>Anelanchier</i> sp.	N	+	-
<i>Caryopteris clandonensis</i>	LA	+	+
<i>C. clandonensis</i>	LA	+	+
<i>Cassia corymbosa</i>	P	+	+(+)
<i>Ceanothus veitchianus</i>	LA	-	-
<i>Cotoneaster</i> sp.	N	+	-
<i>Crataegus prunifolia</i>	LA	-	-
<i>Cytisus burkwoodii</i>	LA	-	-
<i>Daphne mezereum</i>	N	+	+
<i>D. odora</i>	LA	+	+
<i>Euonymus japonicus</i>	N	+	-
<i>Fagus sylvatica</i>	LA	+	NT
<i>Forsythia intermedia</i>	P	+	+(+)
<i>Fraxinus excelsior</i>	LA	+	-
<i>Hamelis mollis</i>	LA	-	-
<i>Hydrangea arborescens</i>	P	+	-
<i>Ilex</i> sp.	N	+	-
<i>Kerria japonica</i>	N	+	NT
<i>Magnolia</i> sp.	N	+	-
<i>Photinia fraserii</i>	N	+	-
<i>Pieris formosa</i>	N	+	NT
<i>Prunus glandulosa</i>	N	+	+
<i>P. sargentii</i>	LA	-	-
<i>P. subhirtella</i>	LA	-	-
<i>Pyrus salicifolia</i>	LA/P	+	-
<i>Rhododendron</i> sp.	P/N	+/-	-(-)
<i>Senecio greyi</i>	P	+	+(+)
<i>S. greyi</i>	P	+	-(-)
<i>Skimmia japonica</i>	N	+	-(-)
<i>Spiraea bumalda</i>	P	+	+(+)
<i>Viburnum carlecephalum</i>	N	+	-
<i>V. farreri</i>	LA/N	+	-(-)

Key : L = Location S = Symptom
T = Sap transmission
+ or - = symptoms or sap transmissible virus, present or absent
NT = Not tested
N = Nursery
P = Park or public gardens
LA = Long Ashton Research Station
Sign in parenthesis indicates positive or negative dsRNA test.

Some effects on growth may have been overlooked because with the exception of those plants grown at Long Ashton, it was not possible to make a reliable comparison with uninfected plants of the same clone. Plants with some form of chlorotic mark or pattern on the foliage, however, a frequent characteristic of plants infected by viruses (Cooper, 1979) formed the bulk of plants surveyed. These ranged from mild mottle (*Senecio greyi*) to severe chlorotic rings and lines and general yellowing (*Forsythia intermedia*). The most detailed survey was that on the genus *Rhododendron* with over 30 samples collected from public gardens or private nursery collections. Most of *Rhododendron* trees or shrubs tested showed no obvious symptom, but a small number showed veinal mottle and leaf distortion (e.g. *R. ponticum* cvs. Elizabeth Bright Red/ Christmas Cheer). The necrotic ring pattern symptom reported by Coyier et al. (1977) on *Rhododendron* in USA and Canada was not observed. Despite numerous attempts using different buffer additives, no virus was isolated from infected plants. No dsRNA was detected in extracts of leaf material from a plant with mottle or distortion symptoms, in a single trial. The failure to isolate virus from plants of many other genera, with symptoms, probably reflects the low

concentrations of virus found in many woody species, as well as the widespread occurrence of inhibitors in such hosts (Fulton, 1966).

Two isolates established from *Daphne nezereum* and *D. odora*, both with conspicuous symptoms, (chlorotic rings and mottle) were tentatively identified from host reactions as cucumber mosaic virus, and were not studied further. Similarly, no further work was done with an isolate obtained from *Prunus glandulosa* with chlorotic rings and blotches which was identified serologically as *Prunus* necrotic ringspot virus. The isolates obtained from *Cassia* (two), *Caryopteris* (two) and *Senecio* (two) formed the basis of the work presented in this thesis.

**SECTION B : VIRUSES IDENTIFICATION AND
CHARACTERISATION**

**CHAPTER 3 : MOSAIC DISEASE OF CASSIA CORYMBOSA
LAM.**

PART A : CASSIA VIRUS NO. 1

Cassia (Family: Leguminosae) is a large genus of mainly tropical trees and shrubs and includes many species of ornamental value. Few viruses have been clearly associated with disease of *Cassia spp.*

The first report was that of Deighton and Tinsley (1958) who described a pale irregular mosaic on the leaves of *Cassia occidentalis*, *C. sophera* and *C. tora* in Sierra Leone, West Africa. In 1959 a similar symptom on *C. occidentalis* and *C. tora* was reported in New Guinea (Velsen, 1961). The causal agent was named cassia mosaic virus (CaMV), and found to have a dilution end point between 1:1000 and 1:2000, thermal inactivation point between 60°- 62°C, and longevity in vitro of less than 168 hours. This virus also had a very limited host range and did not infect any important leguminous food plants.

In India, Mathur and Singh (1972) described a natural infection of coffee senna (*Cassia occidentalis* L.) which was stunted and had light and dark green mosaic patterns on young pinnate leaves. The leaves were small and leathery. An apparently distinct disease of Negro coffee (*C. occidentalis* L.) was described by Verma and Niazi (1974). These authors showed the causal agent to be transmitted experimentally by grafting with an incubation period of 20-30 days, and by an insect; *Aphis solanella*. An electron microscope study from infected leaves revealed that the virus had long flexuous particles. The length of the particles varied from 550-580 nm; the width was 21 nm. The virus reported here resembled the one described by Van Velsen (1961) but was slightly differed in host range. However, on the basis of particle size, host range and mode of transmission, the virus fell into the potexvirus group (Harrison et al., 1971). It was surprising, therefore, that the authors reported transmission by an aphid, *Aphis solanella*.

White clover mosaic virus (WCMV) was also detected on *C. occidentalis* (Joshi et al., 1981) in India. The disease agent was seed-borne in this host but it remained infective in vitro only up to 18-24 hr at room temperature, with a dilution

end-point of 10^{-5} and thermal inactivation point of 60°- 65°C.

Subsequently, tobacco etch virus (TEV) a flexuous rod of 725 nm., length was found to naturally infect *C. tora* in Venezuela (Debrot, 1976), and sicklepod (*C. obtusifolia*) (Demski, 1979). Peanut mottle virus (PnMV) was found on *C. bicapsularis* in Kenya (Bock et al., 1978).

In Brazil, "cerrado" a common native leguminous plant, *C. sylvestris*, showed a mild mosaic on the leaves (Lin et al., 1979). A slightly flexuous rod shaped virus measuring 640 x 15 nm. was isolated and named cassia mild mosaic virus (CaMMV). The virus was considered as a possible member of the carlavirus group according to its properties (Fenner, 1976), although the aphid (*Myzus persicae*) failed to transmit CaMMV from *C. obtusifolia* to healthy *C. obtusifolia* and *C. occidentalis* seedlings. In central Brazil, Lin et al. (1980) described a disease of *C. macranthera* associated with chlorotic spots, vein chlorosis, mild mosaic of leaves and die back symptoms. The cause was identified as CaMMV (Lin et al., 1979) based on symptoms, host range, physical properties in vitro, serology and the reproduction of the symptom on *C. macranthera* seedlings mechanically inoculated with

CaMMV. There is a difference between CaMMV, and CaMV described by Velsen (1961) in host range, as CaMMV does not infect *Chenopodium anaranticolor* while CaMV shows local infection on this host. Moreover, the dilution end point of CaMV is 10^{-3} to 10^{-4} but CaMMV is 10^{-5} to 10^{-8} .

Following this, Paguio and Kitajima (1980) isolated a virus from *Cassia hoffmannseggii* which caused irregular chlorotic blotches and leaf deformation. The virus was tentatively named cassia yellow blotches virus. The virus induced systemic infection in seven plant species belonging to the leguminous and cucurbit families including *C. occidentalis*, *C. obtusifolia*, *Vigna unguiculata* (L) Walp "California Blackeye" and "Clay", and produced local lesions on *Chenopodium anaranticolor*. Leaf dip and ultrathin sections of infected leaves revealed flexuous rod particles of about 845 nm. and cytoplasmic inclusions of the circular and pinwheel types, suggesting that the virus belonged to the potyvirus group.

Recently, in Western Queensland, Australia an isometric virus was detected in the Australian indigenous legume, *C. pleurocarpa* (Dale et al., 1984). Leaves of affected plants showed a vivid yellow blotch symptom. The virus was

called cassia yellow blotch virus (CYBV) and shared many properties with the bromovirus group (Matthews, 1982). These properties included spherical partical morphology 25-27 nm. in diameter, a narrow host range, a sedimentation coefficient of a single component at 85 S and single coat protein of Mr 20,800.

3.01 Symptoms on *Cassia corymbosa* plant

In Great Britain, *C. corymbosa* Lam. is one of only two ornamental *Cassia* spp. hardy enough to be grown outside (Bean, 1970). During a survey on the incidence of virus infection in woody ornamentals, a virus was isolated from a single mature *Cassia corymbosa* growing in an heated glasshouse at the University of Bath. The diseased plant showed a range of "mosaic" type symptoms. Young leaves typically produced chlorotic spotting and flecking with some vein yellowing and leaf distortion. Older leaves showed two types of symptom, one was a persistent severe mosaic and leaf distortion in which leaves developed elongate tips (plate 1). The leaves showed chlorotic blotching often along the main vein or at leaf margins. The second symptom type usually involved generalised mottling of leaves.

Plate 1 Chlorotic mottle, mosaic and leaf distortion
on *Cassia corymbosa* naturally-infected with
CV1



3.02 Isolation of a virus from *Cassia corymbosa* and host range study

The virus was readily isolated from young leaves with symptoms. The leaves were ground 1:3 (w/v) in phosphate buffer pH 7.8 and inoculated to a wide range of herbaceous hosts. The virus isolated from *C. corymbosa* is subsequently referred to CV1.

Host range studies used a "pure" culture obtained from three successive single lesions taken from inoculated leaves of *C. quinoa*, and were repeated at least twice to include both winter and summer conditions. The reactions of herbaceous hosts are summarised in Table 3. Symptomless infection was detected by back inoculation to *Chenopodium amaranticolor* and *C. quinoa*.

The reactions of selected hosts are described further below.

Chenopodium album, *C. amaranticolor*, *C. foetidum*, *C. murale* and *C. quinoa* developed chlorotic or necrotic local lesions on inoculated leaves after 10-14 days (plate 2 a). In *C. amaranticolor* and *C. quinoa* these were followed, in summer, by systemic chlorotic spots and lines

(plate 2 b); other species were not infected systemically.

Nicotiana clevelandii and *N. megalosiphon* developed diffuse chlorotic spots on inoculated leaves and either a systemic chlorotic mottle, or chlorotic lesions, respectively.

Nicotiana tabacum "White Burley" and "Xanthi" produced faint local chlorotic lesions without systemic infection.

Phaseolus vulgaris "The Prince." Inoculated primary leaves showed diffuse chlorotic lesions which often spread along the veins (plate 3 a) followed by systemic chlorotic flecking and vein yellowing on trifoliate leaves (plate 3 b).

Pisum sativum "Montieth". Inoculated leaves were symptomless but vein yellowing, chlorotic spots and mosaic developed on systemically-infected leaves (plate 4). Plants were severely stunted and died within two month of inoculation. Pods on infected plants were twisted and small (plate 5) but the seeds were fertile.

Vicia faba . A chlorotic mottle developed on inoculated leaves, no systemic infection.

Plate 2 (a) Chlorotic local lesions on inoculated *C. quinoa* leaf with CV1.
 (b) Systemic chlorotic spots and lines on *C. quinoa*.

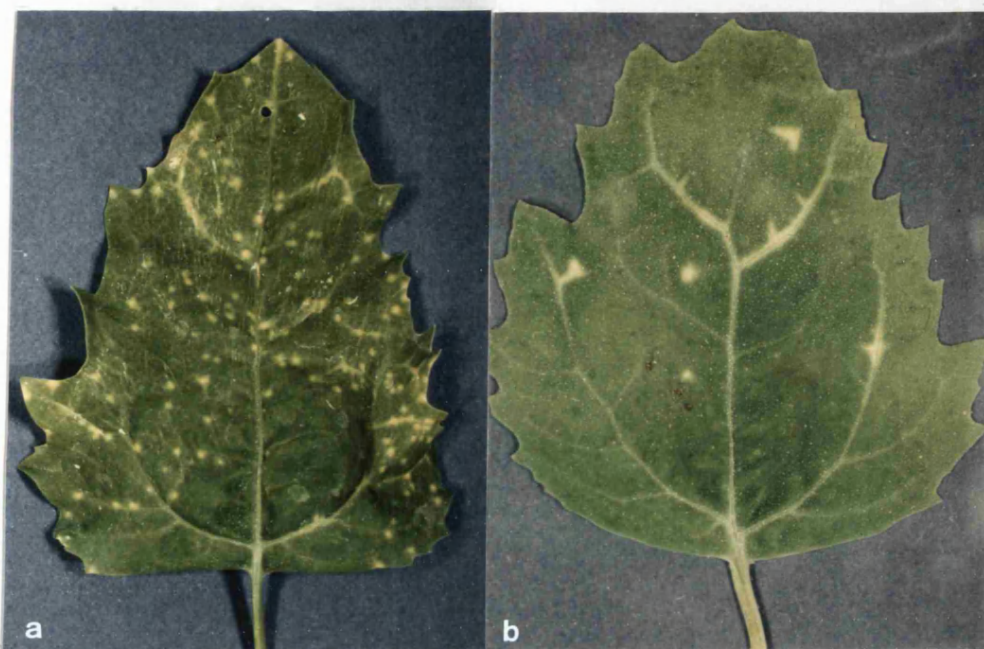


Plate 3 Chlorotic local lesions and vein-yellowing on *Phaseolus vulgaris* mechanically inoculated with CV1 (a) on inoculated leaf
 (b) on uninoculated leaf

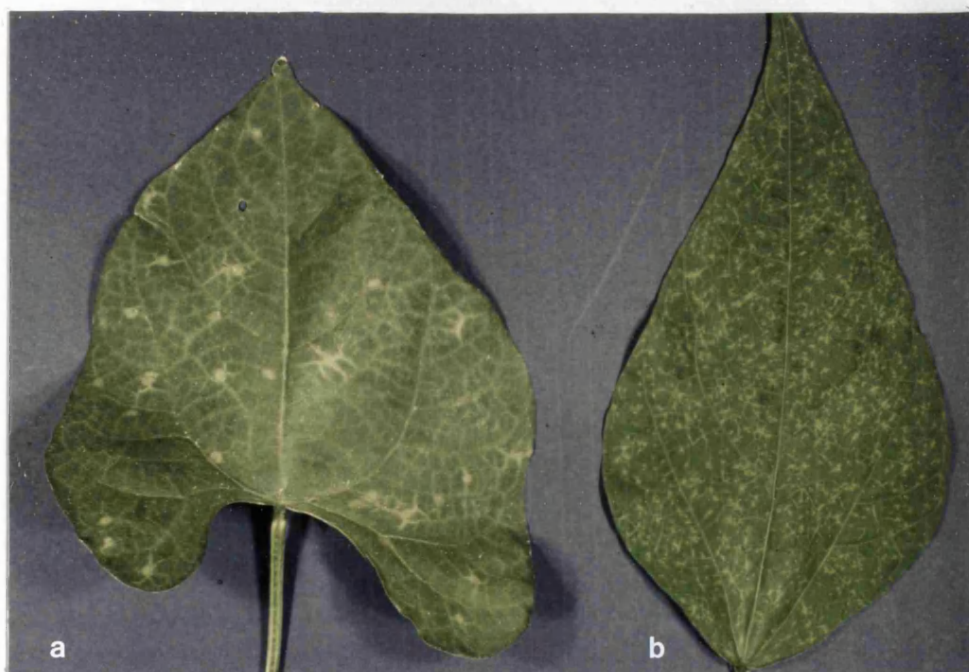
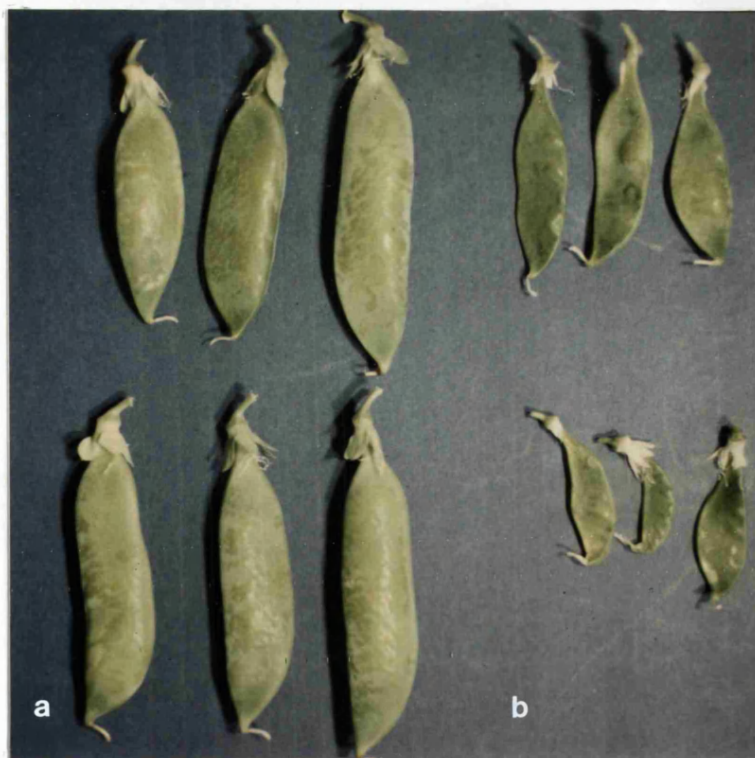


Plate 4 Systemic mosaic in *Pisum sativum*
infected with CV1.



Plate 5 Pods of *Pisum sativum* plants from
(a) healthy plant
(b) infected plant with CV1



Vigna sinensis " Busmita Max "

All leaves were symptomless, but plants showed reduced in growth and vigour

Vigna sesquipedalis developed

systemic necrotic ringspots.

Cassia occidentalis produced a mild

systemic mosaic, while, *C. tora* developed a systemic apical necrosis and the plants died within 3 months (plate 6).

Trifolium repens " White clover "

There was no symptom on inoculated leaves but some vein necrosis was detected on systemic leaves (plate 7).

Trifolium hybridum " Alsike clover "

No symptom on inoculated leaves but systemically infected leaves showed vein mottle, and the plants were stunted.

The following species were not infected by CV1; *Cucumis sativus* "Parisienne Pickling" , *Brassica pekinensis*, *Cassia obtusifolia*, *Nicotiana debneyii*, *N. glutinosa*, *N. rustica*, *N. sylvestris*, *Vigna radiata* type 51, and type HI4, *Glycine max* "Bossier", *Vigna sinensis* "Itta" and type MI35, type MI4, *Wisteria sinensis*, *Pisum sativum* "Onward".

Plate 6 *C. tora* about 2 month old
 (a) mechanically inoculated with CV1
 with apical necrosis.
 (b) healthy plant.



Plate 7 Vein necrosis on systemic *Trifolium*
 rapens cv."White clover" leaves infected with
 CV1



**Table 3 Symptoms induced on herbaceous hosts
by CV1**

<u>Host Plants</u>	<u>Symptom expression</u>
<u>Chenopodium album</u>	CL/O
<u>C. amaranticolor</u>	CL/C,VY *
<u>C. foetidum</u>	CL/O
<u>C. murale</u>	NL/O
<u>C. quinoa</u>	CL/F,VY *
<u>Cassia occidentalis</u>	SI/M
<u>C. tora</u>	SI/N,St,Di
<u>Phaseolus vulgaris</u> cv. "The Prince"	CL,VY/C,F,VY
<u>Trifolium hybridum</u> cv. "Alsike Clover"	SI/M
<u>T. repens</u> cv. "White Clover"	SI/VN
<u>Vicia faba</u>	CL/M
<u>Vigna sinensis</u> cv. "Busmita Max"	SI/St
<u>V. sesquipedalis</u>	NL/NS,F,VN
<u>Glycine max</u> cv. "PM 78-13"	SI/VY,Di
<u>Pisum sativum</u> cv. "Montieth"	SI/VY,CS,M,Di
<u>Nicotiana clevelandii</u>	CL/CM
<u>N. megalosiphon</u>	CL/CS
<u>N. tabacum</u> cv. "White Burley"	CL/O
<u>N. tabacum</u> cv. "Xanthi"	CL/O

Abbreviations : local reactions / systemic symptoms

C = chlorotic or chlorosis	VY = vein-yellowing
N = necrotic or necrosis	VN = vein-necrosis
L = local lesions	D = leaf distortion
M = mottle	St = stunting
F = flecking	S = spots
* = summer only	Di = die-back
O = no infection	
SI = symptomless infection	

3.03 Transmission of the virus from *Cassia* *corymbosa*

Woody plants are notoriously difficult hosts, from which to extract virus (Fulton, 1966). Leaves of many plants, especially woody species, contain tannins which seem to inactivate virus particles by combining with and precipitating them (Gibbs and Harrison, 1976). An important part of these studies on virus diseases of *Cassia*, therefore, were the attempts to improve transmission of virus from *Cassia* to herbaceous indicator plants.

3.03 a) Comparison of virus concentration in original infected *Cassia* plant between season

Transmission of CV1 was achieved at most times of the year, without difficulty. However, during winter it was noticeable that inocula were generally less infective. A log₁₀ dilution series was prepared from young leaves with clear symptoms taken from the original glasshouse grown shrub, during summer and winter. An initial starting dilution of 1:5 (w/v) in 0.05 M phosphate buffer pH 7.5 with 10% insoluble polyvinyl pyrrolidone (Polyclar AT) was used. *Chenopodium quinoa* was used as the assay host and the dilution end-point of both

inocula were compared.

There was no difference in DEP in both samples, whether tested in winter or summer. The infectivity was lost between 10^{-3} and 10^{-4} .

Phenolic compounds which are believed to inactivate virus by forming complexes with them and are often present in woody saps, have been counteracted by adding PVP (Kosuge, 1965). In an attempt to increase transmission efficiency, the effect dilution and polyvinyl pyrrolidone (PVP) additive on infectivity of CV1 in *Cassia* extract was studied.

Young *Cassia* leaves were diced, thoroughly mixed and then divided into four portions of equal weight. Each portion was ground and diluted to 1:5, 1:25 (w/v) in 0.05 M phosphate buffer pH 7.5 with and without 10% insoluble PVP. Before inoculation 4 mg/ml celite was added to each treatment. Four mature leaves on each of four *Chenopodium quinoa* test plants were used according to 4 x 4 Latin square design.

The results are given in Table 4 .

Table 4 Influence of dilution and additive on infectivity of the virus from *Cassia* sap

Treatment	Mean No. lesions/leaf	
	Exp 1	Exp 2
1:5 (w/v)0.05 M buffer	17	17
1:25(w/v)0.05 M buffer	14	12
1:5 (w/v)0.05 M buffer and 10%PVP	13	27
1:25(w/v)0.05 M buffer and 10%PVP	57	92

These results suggested that young *Cassia* leaves contain high concentrations of an inhibitor. Even when the dilution was increased from 1:5 to 1:25 in treatments without 10% PVP, there was little difference ($P>0.05$) in infectivity. However, combination of both high dilution and 10% PVP in extraction buffer significantly ($P<0.05$) increased lesion numbers. This result suggested that dilution was insufficient to dilute out the inhibitor faster than the virus. Presumably the PVP was binding to phenols in the plant sap and preventing them from reducing the virus infectivity of the inoculum (Brunt and Kenten, 1963).

3.03 b) Comparison of different buffers and their molarity on the isolation of virus from *Cassia* leaves

Many viruses have specific requirements for stability and to protect them from inactivation. It is therefore important to know which buffers are favourable for stability if sap transmission is to be achieved.

Three buffers: Tris-HCl, phosphate and HEPES at pH 7.5 and at 0.01, 0.05 and 0.1 M were tested as extracting media. Equal weights of chopped infected young *Cassia* leaves were ground in one of the above buffers at 1:5 (w/v) dilution. Treatments were applied to two sets of 6 *Chenopodium quinoa* plants in 6 x 6 Latin square design.

The results are summarised in Table 5

Table 5 Influence of buffer and molarity on infectivity of *Cassia* extracts

<u>Treatment</u>			<u>Mean no.of lesion per leaf</u>
0.01	M	phosphate buffer	15
0.05	M	phosphate buffer	7
0.1	M	phosphate buffer	6
0.01	M	Tris-HCl buffer	9
0.05	M	Tris-HCl buffer	7
0.1	M	Tris-HCl buffer	6
0.01	M	HEPES buffer	11
0.05	M	HEPES buffer	9
0.1	M	HEPES buffer	14

At a buffer concentration of 0.01 M, phosphate produced a significant ($P < 0.05$) increase in infectivity compared with Tris-HCl and HEPES (Table 5). For phosphate and possibly Tris-HCl, lesion numbers decreased as molarity increased, possibly because CV1 was more unstable at higher molarities (Matthews, 1981). The results with HEPES however did not follow a trend. The results indicated that, under these experimental condition, transmission was favoured by the lowest molarities (0.01 M) of phosphate buffer, although HEPES at 0.1 M appeared equally effective.

3.04 In vitro properties of CV1 isolate

Preliminary experiments established that systemically-infected leaves of *Nicotiana clevelandii* were a good source of the *Cassia* virus, and that the infectivity of the crude sap was stable in 0.01 M phosphate pH 7.5. Therefore, inoculum for determining in vitro properties of CV1 was prepared with *N. clevelandii* extracts diluted, where necessary, in this buffer. Samples were assayed for infectivity on *C. quinoa* plants and lesion numbers counted after 10 or 14 days.

3.04 a) Dilution end point

Serial ten-fold dilutions of crude sap undiluted or diluted in buffer were made down to 1×10^{-5} dilution. Each dilution was assayed on *C. quinoa* in 6 x 6 Latin square design. The infectivity of CV1 was lost between an inoculum dilution of 10^{-4} to 10^{-5} . However, most infectivity was lost in dilutions above 10^{-2} . Average numbers of lesions per leaf (6 leaves) were 156, 116, 39, 13, 2 and 0 for dilutions of 10^{-0} , 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} and 10^{-5} respectively.

3.04 b) Thermal inactivation point

Over two experiments, the TIP of CV1 was between $50^{\circ} - 55^{\circ}\text{C}$.

3.04 c) Longevity in vitro

In sap stored in the dark at a temperature of 18°C infectivity was lost between 20 and 24 hours, most within 8 hours.

3.04 d) Storage

It is always valuable to retain type material of a range of viruses. Although virus isolates can be maintained for long periods by sub-culturing in their respective host, problems of contamination or attenuation may arise. Therefore,

storage methods for the *Cassia* virus were studied using rapid drying over calcium chloride at -20°C and preservation in a deep-freeze at -20°C .

Rapid drying with calcium chloride at -20°C

This method was introduced by Mckinney (1953) and was described by Mckinney and Silber (1968) and Bos (1969).

Each screw-topped bottle was filled with 3 g of anhydrous granular calcium chloride which was covered by a piece of nylon gauze, and then cotton wool which prevented contact of leaf tissue with calcium chloride. The infected *N. clevelandii* leaves were chopped and place over the cotton wool 1 g for each bottle. All the bottles were kept in a deep freeze (-20°C). The individual samples were assayed after increasing periods of time. Within 3 months, the infectivity was completely lost.

Preservation in a deep-freeze

Infected *N. clevelandii* leaves were stored in sealed polyethylene bags and kept in deep freeze (-20°C) for several weeks. Each contained at least 3 g of leaf tissue. After a period of

time, the leaf tissue was assayed on *C. quinoa*. Samples stored frozen remained infective for 6 weeks.

Frozen, infected *N. clevelandii* sap diluted 1:4 (w/v) in 0.01 M phosphate buffer pH 7.5 at -20°C was tested. The sap was pipetted into small eppendorf tubes in 1 ml. aliquots, and kept in a deep freeze (-20°C). Then, each tube was drawn for infectivity assay at intervals and compared with the infectivity of a sap sample inoculated immediately after preparation.

Within the test period mean lesion numbers fell from about 180, before storage, to less than 10 after 5 to 10 months, although variation in test plant susceptibility may now contributed to these figures. About 60% of infectivity was present after 24 hr. at -20 °C., but this fell to 8% after 6 weeks and 2-3% persisted for 5 months. The sample still showed traces of infectivity after 10 months, the longest period tested.

3.05 Stability of virus

Prior to purification it is useful to know the effects of different buffers or additives on the retention of infectivity to avoid unnecessary losses due to aggregation or

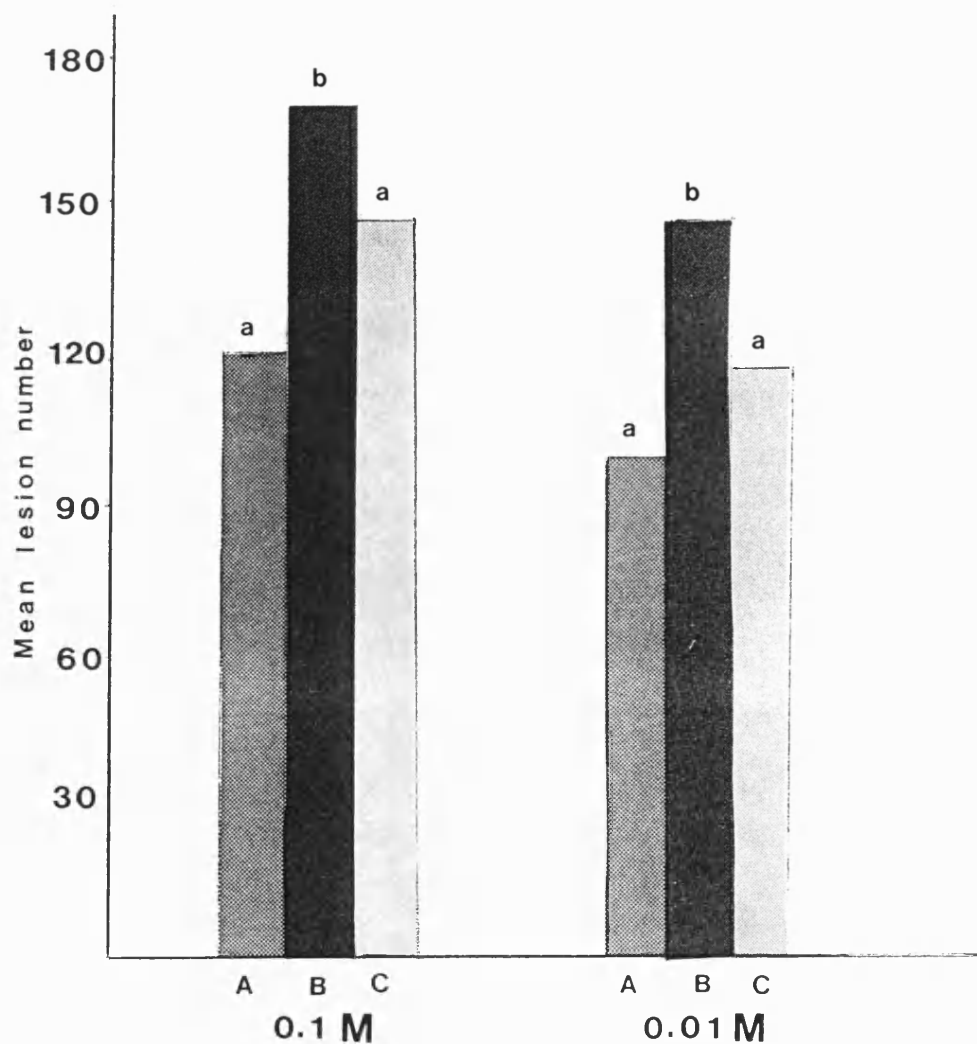
precipitation of the virus, during the purification process. Attention should also be given not only to the ions of the buffers but also to its pH and ionic strength (Scott, 1963).

3.05 a) Influence of different buffers and molarity on stability of CV1

In preliminary tests, 0.01 M phosphate buffer was found to be the most suitable extraction medium to isolate the virus from the infected *Cassia* shrub. However, the conditions which favor stability of purified virus preparations may be different from those needed in crude extracts or partially-purified preparations (Brakke, 1963). Therefore, tris-HCl, HEPES and potassium phosphate buffer at molarity of 0.01 and 0.1 M with pH 7.5 were tested. Inoculum was prepared by grinding weighed equivalent portions of infected *N. clevelandii* leaves in a cold mortar with one of the above buffers at 1:4 (w/v) dilution. The samples were assayed on 2 sets of 6 *C. quinoa* plants in a Latin square design. The results are presented in Figure 2 .

The results indicated that phosphate buffer at both 0.01 M and 0.1 M gave the highest lesion numbers compared with HEPES and Tris-HCl. For a particular buffer, however, there appeared little

Figure 2 Influence of different buffers and molarities on stability of CV1 in *N. clevelandii* sap. (A) tris-HCl (B) phosphate and (C) HEPES buffer at pH 7.5



B and C(0.1M) are significantly different from B and C (0.01M) ($P < 0.025$), bar with different letters are significantly different ($P < 0.025$)

difference between the two molarities although 0.1 M may have been slightly better. As a result of this experiment, phosphate buffer, molarity at 0.1 M was used as extraction medium in further studies.

3.05 b) Effect of different additives on the infectivity and stability of CV1 in *N. clevelandii* extract

The objective in this experiment was to determine whether additives in the extraction buffer would improve stability of the *Cassia* virus. Young leaves from infected *N. clevelandii* were chopped, mixed and separated into six equal weight portions, each of which was ground 1:4 (w/v) in 0.1 M phosphate buffer pH 7.5, alone or containing the following additives: 0.1 M ascorbic acid, 7% bentonite, 7% polyvinyl pyrrolidone (PVP), 2% polyethylene glycol and 0.01 M ethylene diamine tetra acetate (EDTA). Preparations were assayed on *C. quinoa* immediately, and after 4 hr. at 4 °C. The results are shown in Table 6 .

The attempt to stabilize infectivity by incorporating various additives into the extracts showed that 7% bentonite and 2% PEG both failed to increase and stabilize infectivity in extract held at 4° C. for 4 hr. On the other hand, adding 0.01 M ascorbic acid, or 0.01 M EDTA in buffer produced

higher lesion numbers in preparations inoculated immediately compared to phosphate buffer alone. However, after 4 hr. infectivity was reduced significantly ($P < 0.05$) in both treatments. The use of 7% PVP resulted in lower infectivity compared to the control, both after immediate inoculation and after 4 hr. The addition of 0.01 M EDTA appeared to give the highest infectivity so further tests were done with other additives : 0.15% (w/v) sodium mercaptoacetate, 0.01 M diethyl dithiocarbamate (DIECA) (Table 7).

Table 6 Effect of different additives on infectivity and stability of CV1 in *N. clevelandii* extract

Additives	Mean lesion per leaf		% infectivity retained
	0 hr.	4 hr.	
0.1M ascorbic acid	161	49	30
7% bentonite	0	0	0
7% PVP	135	23	17
2% PEG	45	4	9
0.01M EDTA	203	73	36
0.1M phosphate buffer	146	56	38

In Table 7 the additive which gave the highest infectivity when tested immediately after homogenising was 0.15 % (w/v) sodium mercaptoacetate, but the infectivity was lost rapidly within 4 hr. Infectivity was also not

stabilised by adding 0.01 M DIECA. The addition of 0.01 M EDTA to the extraction buffer was successful in both increasing the virus infectivity and stabilising the virus with 55% of the original infectivity being retained after 4 hr. at 4 °C.

Table 7 Effect of different additives on infectivity and stability of CV1 in *N. clevelandii* extract

<u>Additives</u>	<u>Mean lesion per leaf</u>		<u>% infectivity retained</u>
	<u>0 hr.</u>	<u>4 hr.</u>	
0.15% (w/v) sodium-mercaptoactate	291	20	7
0.01 M DIECA	123	15	12
0.01 M EDTA	187	103	55
0.1 M phosphate buffer	165	28	17

3.05 c) Influence of hydrogen ion concentration on infectivity of CV1 in *N. clevelandii*

The pH is usually the dominant factor controlling stability because of its influence on the ionization of groups in both the protein subunits and the RNA that are critical for stability of the virus (Matthews, 1981). Virus particles are more soluble and stable at some pH than others. Many viruses are stable over a rather narrow pH range, and the extract must be maintained

within this range. For every virus there is a pH at which the particles have no net charge (known as the isoelectric point) and at this pH the particles may precipitate. Most viruses have an isoelectric point on the acid side of neutrality, so neutral or slightly alkaline buffers are normally used for extraction (Brakke, 1967).

The infectivity of CV1 was tested at a pH range between 4 to 9. A series of 0.1 M phosphate buffer solutions (without additives) of varying pH values was prepared by grinding equal weights of infected leaf tissue in 0.1 M phosphate buffer (1:2 w/v) then adjusting to the desired pH with 1M HCL or 1M NaOH solution. Sterile distilled water was added to bring the dilution of inoculum up to 1:4 (w/v) before rubbing on to test plants. The inocula were assayed immediately and 4 hr. later.

Table 8 Influence of pH infectivity of CV1 in *N. clevelandii* extract

pH	<u>Mean lesion per leaf</u>		<u>% infectivity retained</u>
	<u>0 hr</u>	<u>4 hr.</u>	
4	0	0	0
5	6	1	17
6	27	13	48
7	27	25	93
8	9	6	67
9	7	4	57

The results in Table 8 indicated that the best stabilization, 93% of original infectivity retained for 4 hr. was with pH 7. When assayed immediately after homogenisation, there was no difference between pH 6 and pH 7, but after 4 hr. incubation there were significantly ($P < 0.05$) fewer lesions at pH 6. At pH 6 the percentage of infectivity retained was about half that for pH 7. Possibly at acidic pH values, the RNA escapes from the protein shell, which is partly degraded, and infectivity is lost (Lyttleton and Matthews, 1958). On the other hand, less than 10 lesions were produced by inoculum prepared in alkaline buffer whether the samples were inoculated immediately or after 4 hr. With alkaline pH, the particles may swell and allow the RNA to escape. The bond between the virus protein and its nucleic acid will be broken and the virus particle will disintegrate (Kaper, 1964; Keeling *et al.*, 1979).

3.06 Propagation of virus for purification

There are several points of practical importance to be considered before the purification of an unknown virus can be accomplished. The choice of host plant for propagating a virus is of critical importance. The extracted sap should be free of inhibitors which might inactivate or

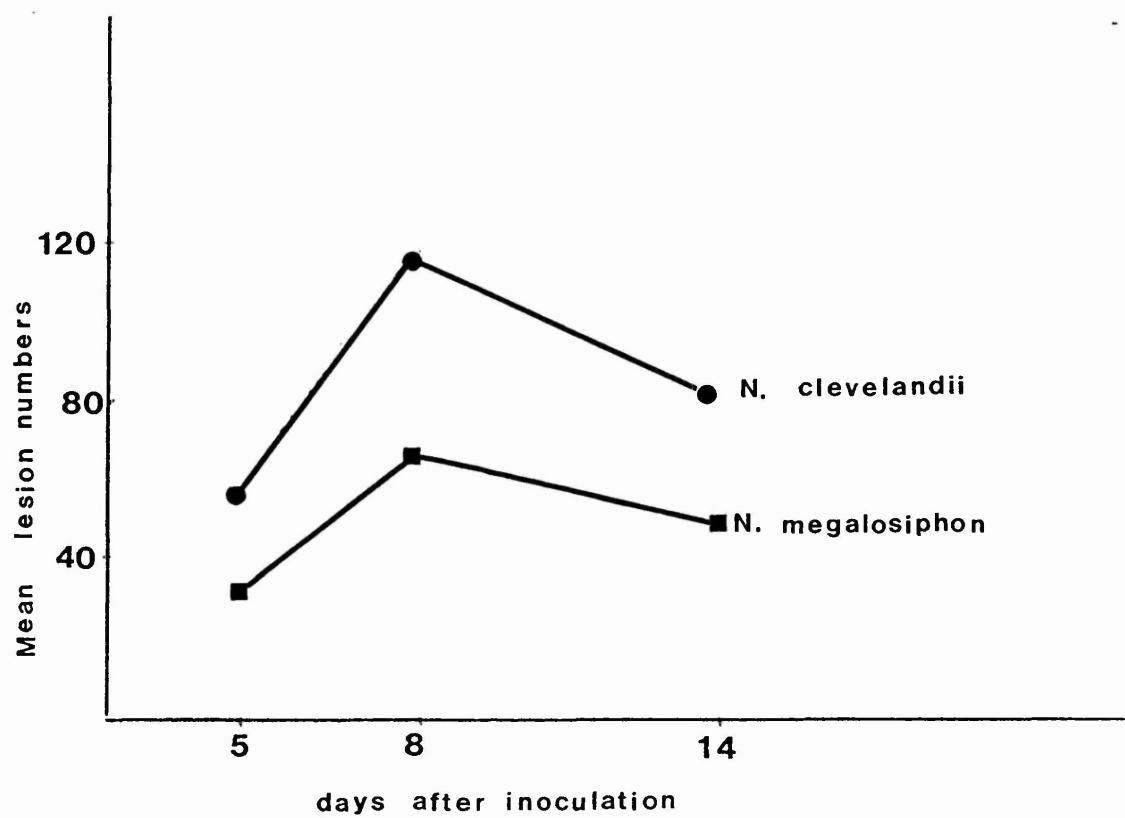
interfere with the virus during purification, and the virus should be easily separable from host material. The time after inoculation that the infected plant material is harvested for purification can also be critical. Hence, preliminary experiments need to be performed.

3.06 a) Comparison of virus concentration in tobacco hosts between *N. clevelandii* and *N. megalosiphon*

The concentration of virus in inoculated *N. clevelandii* and *N. megalosiphon* leaves were assayed on opposite half leaves of *C. quinoa* 5, 8 and 14 days after inoculation, and growth at 17-26°C. The test was done by taking tissue from the inoculated leaves of both host plants at random with a cork borer No.4. Equal weights of tissue from each host were homogenised in two volumes of 0.1 M potassium phosphate buffer, pH 7.5 and equal quantities of celite were added to each inoculum.

The results in Figure 3 showed that the samples from infected *N. clevelandii* leaves gave consistently higher lesion numbers on the test plants than an inoculum from *N. megalosiphon* which was inoculated and harvested after the same period of time. Hence, *N. clevelandii* was chosen to be the

Figure 3 Comparison of CV1 infectivity in inoculated leaves of *N. clevelandii* and *N. megalosiphon*



propagation host for CV1.

3.06 b) Comparison of harvesting time

The experiment was carried out to determine the optimal period, between inoculation and harvest, for maximum virus concentration. *N. clevelandii* plants were inoculated at 5, 8, 11 and 14 days in advance, then the inoculated leaves were sampled randomly using the same method mentioned in 3.06(a) and assayed at the same time on *C. quinoa* in a 4 x 4 Latin square design.

The most suitable time to harvest the inoculated plant material was 8 days after inoculation. Average numbers of local lesions per leaf were 152, 387, 163 and 89 for harvesting time at 5, 8, 11 and 14 days after inoculation, respectively.

3.07 Purification

To identify the causal agent of a disease and study its fine structure in the electron microscope, highly purified preparations should be used. Moreover, the production of high quality antisera also needs a well purified sample. Almost all purification methods involve homogenising, extraction, clarification of the crude sap and precipitation of the virus.

The attempt to purify CV1 was accomplished using *N. clevelandii*, a host in which the virus induces conspicuous local and systemic symptoms. The solvents, chloroform and ether (Francki, 1972) used successfully in clarification procedures with other viruses, were tested. In addition the hydrated calcium phosphate (HCP) method of Fulton (1959) was used.

3.07 Comparison of different clarification and concentration procedures.

3.07 a).1) Clarification with high-speed centrifugation

Chloroform and ether were tested as clarifying solvents for *Cassia* virus purification and compared to HCP and a control treatment which included only the centrifugation steps.

Systemically-infected leaves of *N. clevelandii* were harvested 14-20 days after inoculation. Leaf tissue was divided into equivalent 40 g. lots and each lot was homogenised separately in a Wareing blender with 80 ml of cold 0.1 M phosphate buffer containing 0.01 M EDTA pH 7.5. The samples were blended at low speed for 1 1/2 min and another 1 1/2 min at high speed. After filtering the crude extract through muslin, an equal volume of

ether, or chloroform was added, and the mixtures shaken for 20 min., the control treatment was put in crushed ice. For HCP treatment, before being pressed through muslin, an equal volume of HCP paste was blended with leaf extract. The sample was kept chilled with the control.

All homogenates were then clarified by centrifugation for 20 min. at 10,000 g., after which each supernatant was collected and adjusted to the same volume with buffer before assaying samples on *C. quinoa*.

Equal volumes of each treatment (supernatant) were then centrifuged at 40,000 g. for 3 1/2 hr and the pellets resuspended overnight in 0.25 ml of buffer in each tube. After a brief low-speed centrifugation (4,000 g. for 20 min), each sample was assayed on 2 sets of *C. quinoa* plants in a 4x4 Latin square design. The results are presented in Table 9.

Table 9 Comparison of clarification procedures of CV1

<u>Treatment</u>	<u>Colour of extraction</u>	<u>Degree of clarification</u>	<u>Infectivity*</u>	
			<u>Exp1</u>	<u>Exp2</u>
chloroform	reddish brown	+++	5	8
ether	dark brown	++	2	2
HCP	straw yellow	++++	6	4
control	dark green	+	37	16

+ = poor clarification, ++++ = good clarification
 * = Mean no lesions per leaf

Table 10 Comparison of infectivity after high-speed centrifugation

<u>Treatment</u>	<u>Mean no. lesion per leaf</u>	
	<u>Exp 1</u>	<u>Exp 2</u>
chloroform	2	1
ether	0	2
HCP	9	6
control	5	3

The experiments comparing different purification procedures were divided into two parts: clarification without concentration of virus and clarification with concentration. In the first experiments, without virus concentration (Table 9), HCP treated extracts gave the best clarification and produced the clearest supernatant, a straw yellow colour, while chloroform and ether supernatants were a reddish brown, or dark brown colour, respectively. The control (untreated) supernatant appeared to be dark green, but gave the highest levels of infectivity, in one experiment. In a second series of experiments, (Table 10) the HCP-treated supernatant produced the highest lesion numbers. The results of the first experiments indicated that these clarification agents were generally unsuitable for purification of CV1 as all treatments markedly reduced infectivity. In the second set of experiments with high speed centrifugation, all

lesion numbers were low, including control treatments. This suggested that the high g. centrifugation was having an adverse affect .

Thus, the loss of infectivity may have been due to fragmentation of the particles under the stresses of the gravitational forces. Alternatively, it was possible that as the control treatment was a mixture of both virus particles and host plant material, virus inhibitors present in untreated sap came back into solution during resuspension, and interfered with infectivity assay.

3.07 a). 2) Comparison of concentration procedure between high-speed centrifugation or PEG precipitation

The previous experiment indicated that high-speed centrifugation might be an unsatisfactory way of concentrating the particles, so this method was compared with a modified polyethylene glycol (PEG Mr 6000) precipitation.

Purification was done in the cold using 100 g. of systemically - infected *N. clevelandii* leaves and 200 ml of 0.1 M phosphate buffer with 0.01 M EDTA pH 7.5 as an extraction buffer. The clarification step involved a single low-speed centrifugation at 6000 g. for 15 min.

after which the supernatant was divided into two. One half was mixed with 6% PEG and NaCl added to 0.3 M and the solution stirred in the cold room for 2 1/2 hr. before being centrifuged at 10,000 g. for 20 min. The other half of the supernatant was centrifuged at 40,000 g. for 3 1/2 hr. The pellets from both treatments were left overnight in 0.5 ml phosphate extraction buffer. After a short centrifugation the samples were then assayed on half-leaves of *C. quinoa*.

The mean lesion number from the high-speed treatment in 10 replicate half-leaves was 4, while the PEG precipitation produced a mean of 12 lesions. As a result of this experiment, concentration of the virus particles using PEG seemed a possible alternative to high-speed centrifugation although lesion numbers produced were still low. Nevertheless, further attempts at PEG precipitation were made using a range of concentrations.

3. 07 a).3) Comparison of PEG concentration

Polyethylene glycol has been successfully used for clarification and concentration with several viruses (Gooding and

Hebert, 1967; Leberman, 1966; Van Kammen, 1967). Precipitation of viruses with specific concentrations of PEG (usually PEG 6000) was first described by Hebert (1963). Concentrations as low as 0.5% have been used to precipitate papaya mosaic virus from clarified extracts (Purcifull and Hiebert, 1921). Other concentrations used have been 4% PEG with 0.02 M NaCl for apple stem grooving virus (De Sequeira and Lister, 1969), and 6% or 8% concentration for alfalfa mosaic, turnip mosaic, and red clover mottle viruses (Marcinka, 1971; Clark 1968; Hill and Shepherd, 1972).

The test was performed using the same procedure given in section 3.07 a).2). After removal of host plant material by a low-speed centrifugation, the supernatant was divided into three equal volumes. Then 4%, 6% or 8% PEG Mr 6000 and 0.3 M NaCl was added to each volume. The mixtures were stirred separately for 2 1/2 hr. The virus particles were pelleted by centrifugation at 12,000 g. for 20 min., followed by overnight resuspension in 0.5 ml phosphate buffer. After a brief low-speed centrifugation (4000 g. for 10 min.), each sample was assayed on *C. quinoa*, after diluting 1:2 (v/v) in the same buffer.

**Table 11 Influence of different PEG percentage on
purification procedure**

<u>Treatment</u>	<u>Mean no. lesion per leaf</u>	
	<u>Exp 1</u>	<u>Exp 2</u>
4% PEG	6	4
6% PEG	8	5
8% PEG	13	17

The experiment was repeated once and the results showed that 8% PEG produced the significant highest lesion numbers ($P>0.025$) and of the three concentrations tested, was probably the most suitable concentration to precipitate the virus (Table 11).

Further experiments on CV1 purification, therefore, used this concentration of PEG. Lister and Hadidi (1971) used PEG at the same concentration for routinely precipitating apple chlorotic leaf spot virus.

3.07 a).4 Clarification with PEG concentration

More clarification agents were tested to improve the purification process. The common solvents used in clarification procedures, have included butanol at a concentration of 8-9% for dahlia mosaic virus (Brunt, 1971), and carnation

vein mosaic virus (Hollings and Stone, 1971). Nozu and Yamura (1971) used Triton X-100 (alkyl polyethoxy ethanol) at 10% in the centrifugal purification of tobacco mosaic virus. This detergent lyses chloroplasts, the remains of which are left in the supernatant when the virus is centrifuged.

Carbon tetrachloride emulsification was used by Fribourg and de Zoeten (1970) for potato virus A, and also has been used for a strain of red clover vein mosaic virus (Bos et al., 1972), potato mop top virus (Kassanis et al.., 1972), and robinia mosaic virus (Schmelzer, 1971). Bentonite has been used as an adsorbent for clarifying extracts of necrotic fleck disease of lily (Civerolo et al., 1968), and apple stem grooving and chlorotic leafspot viruses (Uyemoto and Gilmer, 1971).

All four above solvents were tested as clarifying agents and compared with an untreated control. After homogenisation of 200 g. of systemically-infected *N. clevelandii* leaves in 400 ml of 0.1 M phosphate buffer containing 0.01 M EDTA pH 7.5, using a blender, the extract was filtered through two layers of muslin. Then, 8.5% butanol, 10% Triton X-100, 10% carbon tetrachloride or 15% of 40 mg/ml bentonite solution, prepared according to Dunn and Hitchborn (1965), were added to an equal volume

of aqueous extract except for the control, which was untreated. Each mixture was shaken gently for 30-40 min. at 4°C. whereas, the control treatment was kept unshaken. For the subsequent step, all treatments were spun at 10,000 g. for 20 min. and the supernatants collected; the colour and degree of clarification were noted. After 8% PEG and 0.3 M NaCl were mixed into each treatment and stirred for 2 1/2 hr., virus was precipitated by centrifugation at 12,000 g. for 20 min. To compare different treatments samples were an assayed on *C. quinoa*. After the pellets had resuspended overnight (4 °C.) in 0.5 ml. phosphate extraction buffer, and the solution was given a low-speed centrifugation at 4000 g. for 10 min., the treaments were assayed in a 5 x 5 Latin square design.

**Table 12 Effect of different clarification
 methods on the infectivity of CV1 in *N.*
 clevelandii extracts**

<u>Treatment</u>	<u>Degree of</u> <u>clarification</u>	<u>Colour</u>	<u>Infectivity*</u>
butanol	++++	light green	0
Triton X-100	+	dark green	4
bentonite	++++	straw yellow	0
carbon tetrachloride	+	dark green	7
control	+	dark green	13

+ = poor clarification, ++++ = good clarification
* = Mean no. lesions per leaf

From the result presented in Table 12, it appeared that none of the solvents tested was able to remove host material without causing a loss of infectivity. The supernatant from butanol and bentonite treatment produced a high degree of clarification but no local lesions on the test plants. On the other hand, Triton X-100, carbon tetrachloride and control gave very poor clarification with a dark green supernatant but produced few lesions : 4, 7 and 13, respectively.

The partially-purified control sample was examined under an electron microscope with 2% PTA pH 6.5. A few virus-like particles with flexuous shape and a range of sizes were observed, with no clear modal length. The different lengths of particles might have been caused by particle damage during homogenisation in blender. Many of the very long particles such as those of closteroviruses, are likely to break under mechanical stresses during extraction and purification (Till and Shepherd, 1967; Bar-Joseph et al., 1970).

3.07 b) Chemical supplements

The addition of suitable chemicals to the extraction medium, in order to stabilise infectivity, can be critical when purifying an

unstable virus. According to previous experiment, 0.01 M EDTA added to phosphate buffer was successful in increasing infectivity and partially stabilising the particles within 4 hour period. However, purification can be prolonged, so the presence of EDTA in the extraction medium was compared with phosphate buffer without an additive, in a purification scheme over 2 days.

The experiment based on that from section 3.07 a).3, used 100 g. infected *N. clevelandii* without clarification before low-speed centrifugation. The virus was precipitated by 8% PEG and 0.3 M NaCl and 20 min. centrifugation at 12,000 g. The pellets were resuspended overnight in buffer, with and without EDTA, followed by a short centrifugation 4000 g. for 10 min. to get rid of all debris left in partially-purified samples. These two treatments were assayed by inoculation to opposite half-leaves of *C. quinoa* seedlings.

The mean number of lesions per half-leaf were 18 and 7 (10 replicates) for buffer only and buffer with EDTA, respectively. Buffer without EDTA gave higher lesion numbers and was significantly better ($P < 0.025$) than the sample in buffer with EDTA.

This result contrasted with the preliminary experiment on the effect of EDTA on virus stability. However, it is possible that the consistently low lesion numbers were responsible for the erratic results and make conclusions difficult and unreliable.

3.07 c) Purification without using blender

Some viruses are susceptible to breakage during mechanical blending, so to avoid shearing during blending 50 g. infected *N. clevelandii* leaves were frozen overnight. The plant tissues could then be homogenised more efficiently before being hand-ground in a cold mortar in 250 ml. of 0.1 M phosphate buffer at pH 7.5 (without an additive). Eight percent PEG with 0.3 M NaCl was used to precipitate the virus.

The centrifuge tubes were divided into two groups. The virus pellet after PEG precipitation was left to resuspend for 1 hr. or overnight (4°C.) in 1 ml phosphate buffer before the samples were assayed on *C. quinoa*.

The partially-purified preparation, resuspended for 1 hr. was moderately infective whereas resuspending the pellet overnight caused an

infectivity loss of about 78%. The mean of lesion numbers per leaf were 32, and 7, respectively for 1 hr. and overnight resuspension.

The absorption spectrum and the ratio of nucleic acid:protein (260:280 nm.) of the sample resuspended overnight, was measured after the preparation was diluted 1:100 in phosphate buffer. The 260:280 ratio was 1.9 which is abnormally high for most viruses with rod-shaped particles. However, some closteroviruses have 260:280 ratio at 1.8 (Lister and Bar-Joseph, 1981). Infectivity of the 1/100 dilution was very low (2-3 lesions/leaf) possibly through viral aggregation or low virus concentrations.

The low infectivity of CV1 in many previous experiments may have been caused by the damage of viral particles during blending. In this experiment, leaving the pellets in resuspending buffer overnight may have increased aggregation, or virus degradation.

3. 07 d) Study of suitable resuspending time

In order to improve purification further, the optimum resuspension time for CV1 was determined. The pellets from PEG precipitation were

dissolved in 0.1 ml phosphate buffer pH 7.5 and kept at 4 °C. The assay was done on the whole leaves of *C. quinoa* in a 4 x 4 Latin square design after resuspension intervals of 1, 2, 3, and 4 hr.

Table 13 Infectivity of partially-purified preparations after different resuspending time

<u>Resuspending time</u>	<u>Mean no. lesion per leaf</u>
1 hr.	23
2 hr.	41
3 hr.	17
4 hr.	5

The results suggested that a suitable time to leave virus pellets resuspending was 2 hr. at 4 °C. which gave significantly ($P < 0.05$) higher lesion numbers on test plants than other times (Table 13). The 4 hr. resuspension period produced very few lesions. This low infectivity might due to a tendency of the particles to aggregate in suspension (Lister and Bar-Joseph, 1981). In further experiments, the pellets from PEG precipitation were routinely dissolved for 2 hr. in buffer, at least until a suitable additive could be found to prevent the aggregation.

3. 07 e) Influence of different molarities of resuspending buffer on infectivity of CV1

In this study, pellets from a standard PEG precipitation [section 3.07 a).3] were resuspended in phosphate pH 7.5 buffer of different molarities from 0.001-0.1 M. After 2 hr. and a brief low speed centrifugation all the samples were diluted 1:3 (v/v) before being assayed in a 5 x 5 Latin square design on *C. quinoa*.

Table 14 Influence of different buffer molarity on infectivity of partially-purified preparation

<u>Treatment</u>	<u>Mean no. of lesion per leaf</u>
0.1 M phosphate buffer	4
0.05 M phosphate buffer	31
0.01 M phosphate buffer	32
0.005 M phosphate buffer	29
0.001 M phosphate buffer	59

Table 14 indicated that at 0.001 M phosphate buffer produced the highest mean lesion number. In contrast to, the 0.1 M treatment produced very few lesions. This experiment suggested that the lower ionic strength of buffer increased the infectivity of virus significantly ($P > 0.05$), possibly by reducing aggregation.

3.07 f) Study of additive chemicals
in resuspending buffer

As a virus is purified, stabilizing substances are removed and hence the environment for stability will change. Some stabilizing substances may be replaced by the addition of specific ions, such as magnesium and calcium in the case of brome mosaic virus (Brakke, 1963). Inouye (1974), Bar-Joseph and Smookler(1976) added 0.1% (v/v) Triton X-100 in resuspending buffer for carnation necrotic fleck virus (CNFV) purification.

In this study, additives such as 0.01 M EDTA, 0.1% Triton X-100, 0.05 M urea and 0.01 M magnesium chloride added to 0.001 M phosphate buffer pH 7.5 were compared. The suspension time was also compared between 2 hr. and overnight at 4 °C.

**Table 15 Influence of chemical in resuspending
buffer on infectivity of CV1**

<u>Treatment</u>	<u>Mean no. lesion per leaf</u>	
	<u>2 hr.</u>	<u>overnight</u>
0.05 M urea	23	8
0.01 M magnesium chloride	19	3
0.1% Triton X-100	7	0
0.001 M EDTA	22	6
control(buffer only)	37	15

The chemicals added to resuspending buffer, gave no evidence of improving infectivity at both times tested. Especially with 0.1% Triton X-100, the lesion numbers were the lowest after 2 hr resuspension, while the sample was no longer infectious after overnight resuspension. Resuspension in buffer with 0.05 M urea, 0.01 M magnesium chloride or 0.001 M EDTA gave similar lesion numbers but these were significantly ($P < 0.05$) lower than those of buffer only. For this reason, it was considered worthwhile exploring other methods to achieve purification.

3.07 g) Study of resuspending virus pellets in different buffer volume

The experiment was performed to find out how the volume of buffer used for resuspension of the pellet after standard PEG precipitation affected the infectivity of the virus solution. Different volumes of 0.001 M phosphate buffer from 0.5 ml to 2.5 ml were applied to virus pellets obtained from equivalent volumes of virus-containing supernatant. Infected *N. clevelandii* leaves were used in this test and two different resuspending times: 2.1/2 hr and overnight, both at 4 °C. The experiment was repeated using an equal starting weight of whole infected *Pisum sativum* plants.

With overnight resuspension there appeared little difference in lesion numbers ($P>0.05$) with 1.0, 1.5, 2.0 or 2.5 ml buffer, although lesion numbers from pea material resuspended in 0.5 ml were significantly reduced ($P<0.05$) (Table 15).

Table 15 Effect of different volumes of
resuspending buffer on infectivity

Volume (ml)	Mean no lesion per leaf			
	2 ¹ / ₂ hr.resuspended		overnight resuspended	
	Pea	<i>N. clevelandii</i>	Pea	<i>N. clevelandii</i>
0.5	31	9	6	3
1.0	72	31	15	2
1.5	36	10	19	1
2.0	51	30	25	4
2.5	52	21	22	3

For virus pellets resuspended for 2 ¹/₂ hr results suggested dilution improved the virus infectivity particularly with 1 ml, although results were variable for both pea and *N. clevelandii*. Lesion numbers were generally higher when pea tissue was used as the virus source.

The earlier results which suggested increased infectivity with shorter (2hr.) resuspension periods, were confirmed here. The effect of dilution may be to reduce particle aggregation.

3.07 h) Differential comparison of
host plant material for purification

3.07 h).1) *N. clevelandii* and
Phaseolus vulgaris "The Prince"

Both host plants were considered because they gave consistent systemic infection. In addition bean could be easily and quickly grown from seeds in large quantities. Each 100 g. of inoculated and systemically infected bean leaves were harvested after 14 days, whereas, *N. clevelandii* was left until 14-20 days after inoculation. HCP 1:1 (w/w) was applied as a clarification agent before low-speed centrifugation. The purification scheme was based on that given in section 3.07 a).1). The two purified samples were assayed on opposite half-leaves of *C. quinoa*.

Table 16 Comparison of host plant material for purification

<u>Host</u>	<u>Supernatant colour</u>	<u>Mean no lesion. 10 replicates</u>
<i>Phaseolus vulgaris</i> "The Prince"	murky dark green	1
<i>N. clevelandii</i>	straw yellow	10

This experiment demonstrated that purification using *N. clevelandii* resulted in higher lesion numbers than bean. Furthermore, the supernatant from bean was murky and dark green even after extracts were clarified with HCP. It is possible that the host material and pigments from bean were not satisfactorily adsorbed by the HCP gel. The colour with *N. clevelandii* extracts was lighter with less host material present. From this experiment, there was little indication that *Phaseolus vulgaris* "The Prince" could be used for purification. The generally low lesion numbers using HCP/high-speed centrifugation found earlier [3.07 a).1] were confirmed in this experiment.

3.07 h).2) *N. clevelandii* and
Pisum sativum "Montieth"

100 g. of whole systemically-infected pea plants with strong mosaic symptoms, or *N. clevelandii*, were harvested 3 weeks after inoculation. The leaves were frozen overnight and then homogenised by hand-grinding with 1:5 (w/v) 0.1 M phosphate buffer pH 7.5. Clarification was by low-speed centrifugation and PEG precipitation was used to concentrate the virus [3.07 a).3]. The time allowed to resuspend the pellets was 2 1/2 hr.

The samples were applied to opposite half-leaves of 10 *C. quinoa* seedlings. The lesion numbers obtain when *N. clevelandii* was the source of virus was nearly 3 times lower than the sample from pea, although the supernatant colour indicated the same degree of clarification (Table 17).

Table 17 Comparison of host plant material for purification

<u>Host</u>	<u>Supernatant colour</u>	<u>Mean no. lesion</u> <u>10 replicates</u>
<i>N. clevelandii</i>	green	14
<i>Pisum sativum</i> "Montieth"	light green	36

Thus, for future work, *Pisum sativum* "Montieth" was considered a better source for purification. Furthermore, pea seeds were easier to germinate and the plants took a shorter time to reach inoculation size. In addition, the whole plant could be harvested as the virus was systemic and the tissues soft, and relatively high concentrations of virus were present.

3.08 Transmission of plant viruses.

Plant viruses apparently cannot enter intact plants but gain access through wounds, which are sometimes produced naturally or by other

organisms that may carry the viruses. The property of transmissibility is a fundamental characteristic of a virus. In this study transmission by the aphid *Myzus persicae*, grafting and seed, was investigated.

3.08 a) Seed transmission

In many virus/host combinations where seed transmission occurs the proportion of seeds infected is usually small, but in some, for instance, tobacco ring spot virus in soybean, most of the seeds may be infected (Desjardius et al., 1954). Not all viruses cause symptoms in their host when they infect through the seed. Thus, selection by visual inspection of apparent virus-free parent plants may often be an unreliable means of avoiding seed-borne virus spread. Therefore, a test is needed, to establish the presence of seed-borne virus.

Seeds from infected *N. clevelandii* were sown at the rate of 50 per pot in Levington compost and gibberellic acid solution (100 p.p.m.) was sprayed for 2-3 days after sowing. Seedlings were picked randomly and tested for seed-borne virus by sap inoculation of phosphate buffered extracts from the leaves of seedlings to a group of *C. quinoa* plants.

After 14 days, all assay plants showed no symptoms.

In a further test about 300-500 *N. clevelandii* seeds from plants infected with CV1 were ground in a mortar with 3 ml of 0.1 M phosphate buffer pH 7.5. The seed homogenate was then rubbed on to leaves of *C. quinoa* plants after adding celite. No symptom was observed on any test plants.

Seeds harvested from infected *N. clevelandii* were homogenised and a sap sample was stained in a few drop of 2% (w/v) solution of PTA pH 6.5 when the preparation examined under electron microscope. No particles were detected in seed preparations.

The results indicated that CV1 was not seed-borne in *N. clevelandii*.

3.08 b) Graft transmission

A group of 10 healthy *Cassia corymbosa* seedlings about 4 month old were tested by flap grafting method (Wallace, 1947; Garnsey and Whidden, 1970). Young infected *Cassia* leaves from the original shrub were cut in a rectangular shape c.5 x 8 mm. in size, sliced across the veins then inserted under an equal sized flap cut in the bark

of healthy plants. To hold tissues together firmly until they united, and to prevent water loss, the graft union was wrapped with Parafilm strips. These plants were then kept in a glasshouse with an temperature range of 23°-28°C. and supplementary light to encourage growth. After 8 weeks, the young leaves of nine flap-grafted *Cassia* showed chlorotic spots, flecks and lines (see plate 9), whereas on older leaves, chlorotic mosaic and mottle (especially at leaf margins) and leaf distortion was observed. Symptoms produced closely resembled those seen in the original naturally-infected shrub.

3.08 c) Insect transmission

An advantage of vector transmission tests, is that they indicate the potential for spread of the virus from the host material, and thus have value in broader epidemiological studies. The peach potato aphid (*Myzus persicae* Sulz.) is probably the most important vector in temperate regions of the world because it transmits about seventy viruses. As a result of this, *Myzus persicae* was tested for ability to transmit CV1 in non-persistent, semi-persistent and persistent manner.

Non-persistent : About 100 adult, wingless aphids were transferred to a plastic petri-dish with a cover, then sealed with Parafilm. This petri-dish was left for 1 hour away from the sun, in order to starve the aphids. Later at least 10 aphids were moved to each separate leaf of infected *N. clevelandii*, and allowed to feed on a diseased plant for 5 min before transferring 10 aphids to each healthy *N. clevelandii* plant. Inoculation period was about 1 hour.

Semi-persistent : After 1 hr starving period, the aphids were fed on disease plants for at least 16 hr. Then, they were transferred to healthy test plants for about 1 hr inoculation.

Persistent : Aphids were handled in the same way as for non-persistent virus transfer, except that no starvation period was given before acquisition, which took 3 days on infected *N. clevelandii* plants. Then, the 10 aphids were carefully transferred to each healthy *N. clevelandii* plants and left for a further 24 hr. The aphids were then transferred three times to fresh healthy *N. clevelandii* to allow for a long latent period before transmission was possible.

"Pyrethroid" insecticide was sprayed over the test plants to kill the aphids after the inoculation period was completed. These experimental plants were kept under an insect-proof cage in the glasshouse and grown on for symptom observation. Each *N. clevelandii* plant was inoculated back to a *C. quinoa* seedling to test if any virus was present after 6 weeks.

Myzus persicae failed to transmit CV1 in every case : non-persistent, semi-persistent or persistent transmission. There was no systemic symptom observed on inoculated *N. clevelandii* or any local lesion on *C. quinoa* seedlings after back testing the leaves from inoculated plants.

The results suggested that *Myzus persicae* was unable to transmit CV1 from *N. clevelandii* to *N. clevelandii*.

3.09 Return inoculation of CV1 isolate to *Cassia corymbosa* seedlings.

Owing to the low yields of purified virus, the attempts to return the disease agent to healthy *Cassia corymbosa* seedlings was demonstrated, using a culture taken from a third successive single lesion isolate in *C. quinoa*. The inoculum was 1:3 (w/v) dilution in 0.1 M phosphate

buffer pH 7.5 and a pinch of celite was added before the inoculum was rubbed on leaves of 5 pre-darkened *Cassia* seedlings aged 6 weeks. After 2 weeks in a glasshouse with a temperature range of 17°-35°C 4 of the seedlings started to show slight mottle on leaves, and by 4 weeks growth reduction of infected plants was clearly visible, compared with healthy seedlings of the same age (plate 8). The leaf symptoms which developed, closely resembled those seen in the original naturally infected shrub, and were identical to those seen in seedlings flap-grafted with tissue from the original shrub (Section 3.08 b). The leaves showed a range of "mosaic" symptoms including chlorotic spots, flecks and lines, yellowing, marginal chlorosis. Some of the older leaves were distorted and malformed shape (plate 9).

The virus isolated from inoculated plants produced typical symptoms of CV1 on *C. quinoa* and other hosts.

Further indication that the inoculated plants contained the same virus as the original *Cassia* shrub were obtained by dsRNA analysis (see Section 3.10) and electron microscopy (Section 3.11).

Plate 8 Healthy *C. corymbosa* (left) and plant (right) of the same age, inoculated with CV1.



Plate 9 Young leaves of *C. corymbosa* seedlings, 4 weeks after flap grafting, either with a single lesion isolate of CV1 (left) or with tissue from the naturally infected shrub (right)

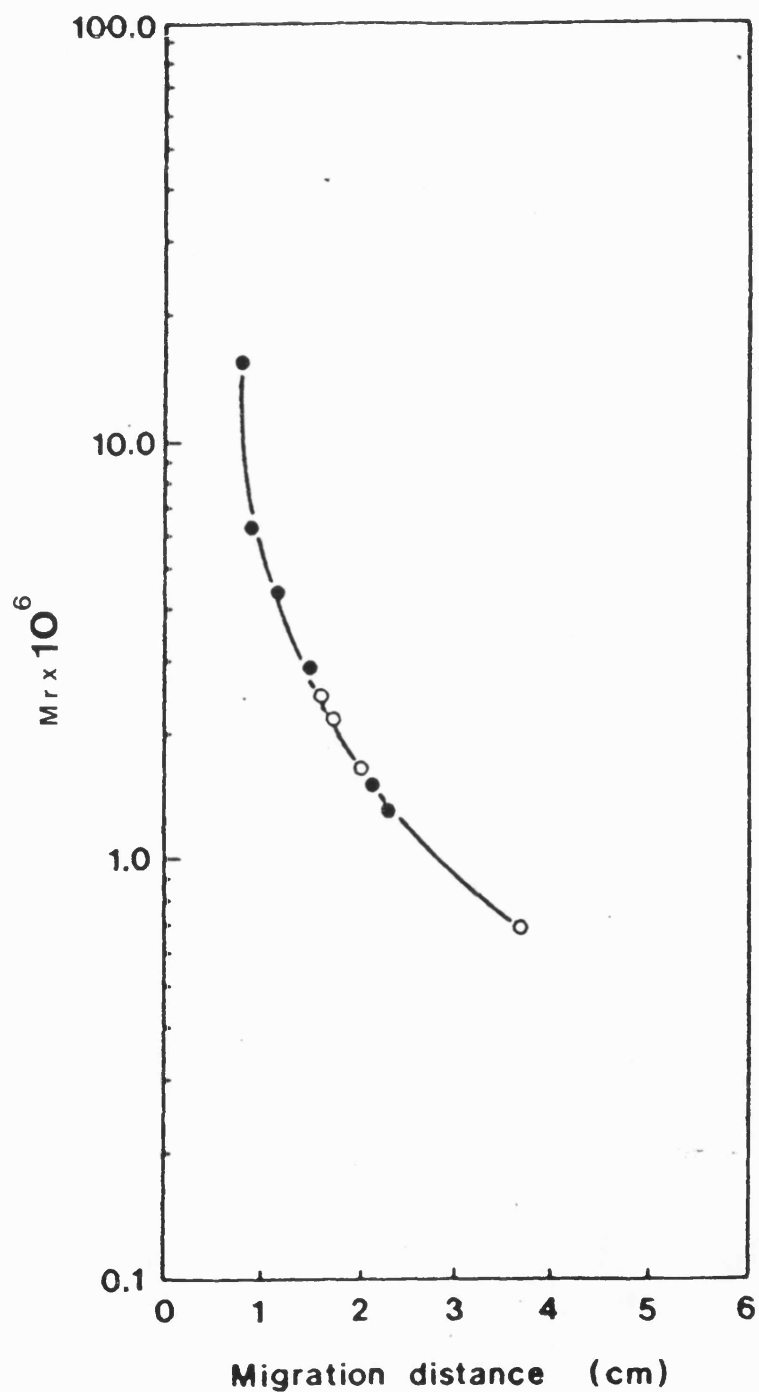


3.10 Double-stranded RNA analysis

In this experiment, parallel dsRNA extractions were conducted using leaf material from 30 g. of infected *N. clevelandii* or *Phaseolus vulgaris* cv."The Prince". When dsRNA samples were precipitated with absolute ethanol, following CF-11 cellulose chromatography, large amount of dense, mucilaginous material co-precipitated with both samples. Dried pellets were resuspended in 150 μ l electrophoresis buffer and 10% glycerol before being applied to 6% polyacrylamide gels. The electrophoresis was performed at 5-6 mA/tube for 3-4 hr.

Deoxyribonucleic acid (DNA) was present on the gel as a contaminant even though the cellulose technique is reportedly specific for dsRNA. A DNAase treatment step was essential because some high relative molecular mass dsRNA bands comigrated with host DNA. After DNAase digestion two bands were detected in bean extracts. Using defined Mr markers a curvilinear relationship was established between electrophoretic mobility and log Mr (Figure 4). Using Fig 4 relative molecular mass (Mr) estimates for the two dsRNA bands were 13.0×10^6 and 1.7×10^6 . In the extract from *N. clevelandii* only one dsRNA species was detected

Figure 4 Electrophoretic mobility of dsRNA as a function of log relative molecular mass.
 ●—● - bacteriophage DNA
 ○—○ - CMV (isolate from lettuce)



with an estimated Mr of 13.0×10^6 .

In order to support the causal association between CV1 and the disease in the original shrub a dsRNA extraction was repeated using 30 g. of *Cassia* leaves with clear symptoms, from the original shrub, and 30 g. from the artificially infected *Cassia* seedlings. The dsRNA was purified as mentioned in Section 2.14, but as the extracts were mucilaginous and the cellulose columns rapidly became blocked, they were emptied into a 5 ml centrifuge tube and washed by shaking gently in 17% ethanol in STE buffer. After centrifugation (6000 g/10min.) the supernatant was discarded and the washing repeated 3-4 times after which the column was repoured and purification continued as described by Hicks et al., (1988).

For the original *Cassia* shrub, three "major" bands (by concentration) of dsRNA were consistently detected, as well as faint "minor" bands. Relative molecular mass estimates for the "major" bands (using a Hind III lambda standard) over four experiments in which dsRNA and fragments were run on the same gel, indicated $13-15 \times 10^6$ for the highest Mr species (band 1) and $1.2-1.3 \times 10^6$ and $0.4-0.5 \times 10^6$ for the other species (band 2 and 3 respectively). Band 2 was usually in highest concentration as judged by the intensity of

fluorescence under UV.

For the inoculated *Cassia* sample, band 1 and 3 were detected but not band 2 which was clearly visible only in samples from the mature shrub (plate 10).

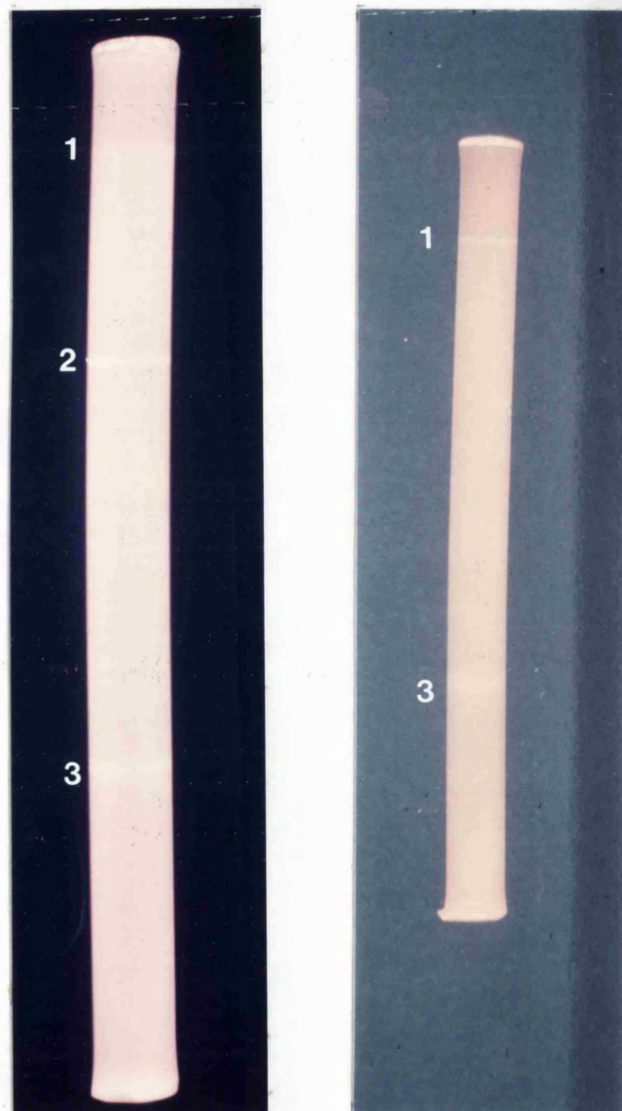
Apparently virus-free *C. corymbosa* contained no detectable dsRNA.

3.11 Electron microscopy

Negative staining of tissue extracts

Leaf tissue of infected *N. clevelandii* and *Cassia* were examined for virus-like particles in a JEOL 100 CX electron microscope at 80 KV. The following stains (all at 20g/l) were tested; potassium phosphotungstate adjusted to pH 5.0, 6.5 or 8.0 with KOH; ammonium molybdate pH 5.0; uranyl acetate and uranyl formate (both unadjusted). In preliminary tests, fewer particles were seen in leaf preparations made by "dips" rather than "squashes" so the latter were generally used. Different types of tissue were also compared including whole leaf squash, epidermal strips and leaf vein tissue. Small pieces of leaf tissue were ground in sterile distilled water on a slide and the

Plate 10 Ethidium bromide stained polyacrylamide (6%) gels showing dsRNA extracted from *C. corymbosa* infected with CV1
(a). naturally- infected
(b). infected with the single lesion isolate.



sap added to a filmed microscope grid. After 5-10 min. the grid was drained and then washed carefully in stain when it was drained and dried before examination. Some samples were ground directly in stain, with the addition of a wetter (0.01% bacitracin). Particle sizes were estimated by comparison with negatively stained catalase crystals (Wrigley, 1968).

Examination of negatively - stained leaf "squash" preparations from infected *N. clevelandii* showed the presence of apparently fragile, flexuous, filamentous particles mostly as single particles (plate 11 a), but occasionally in "tangled" clumps (plate 11 b). The flexuous particles were detected in "squashes" prepared from both epidermal strips and vein tissue, the latter being the most consistent source of particles. The length of individual filaments in these preparations was highly variable and ranged from 150 nm. to 2168 nm. The range of particle lengths was similar regardless of the stain (or pH) used or whether the samples were fixed in 1% glutaraldehyde.

Most particles (72%) were under 1000 nm. in length and most of these were 500-800 nm. Out of 41 particles 1000 nm. or over, 14 were 1000-1200 nm, 17 were 1300-1500 nm, 8 were 1600-1900 nm. and

Plate 11 Electron micrograph of leaf squash
preparation of CV1 from *N. clevelandii*
stained in 20 g/l PTA, pH 6.5.
(a). single flexuous rods found in veinal
squash

(a)

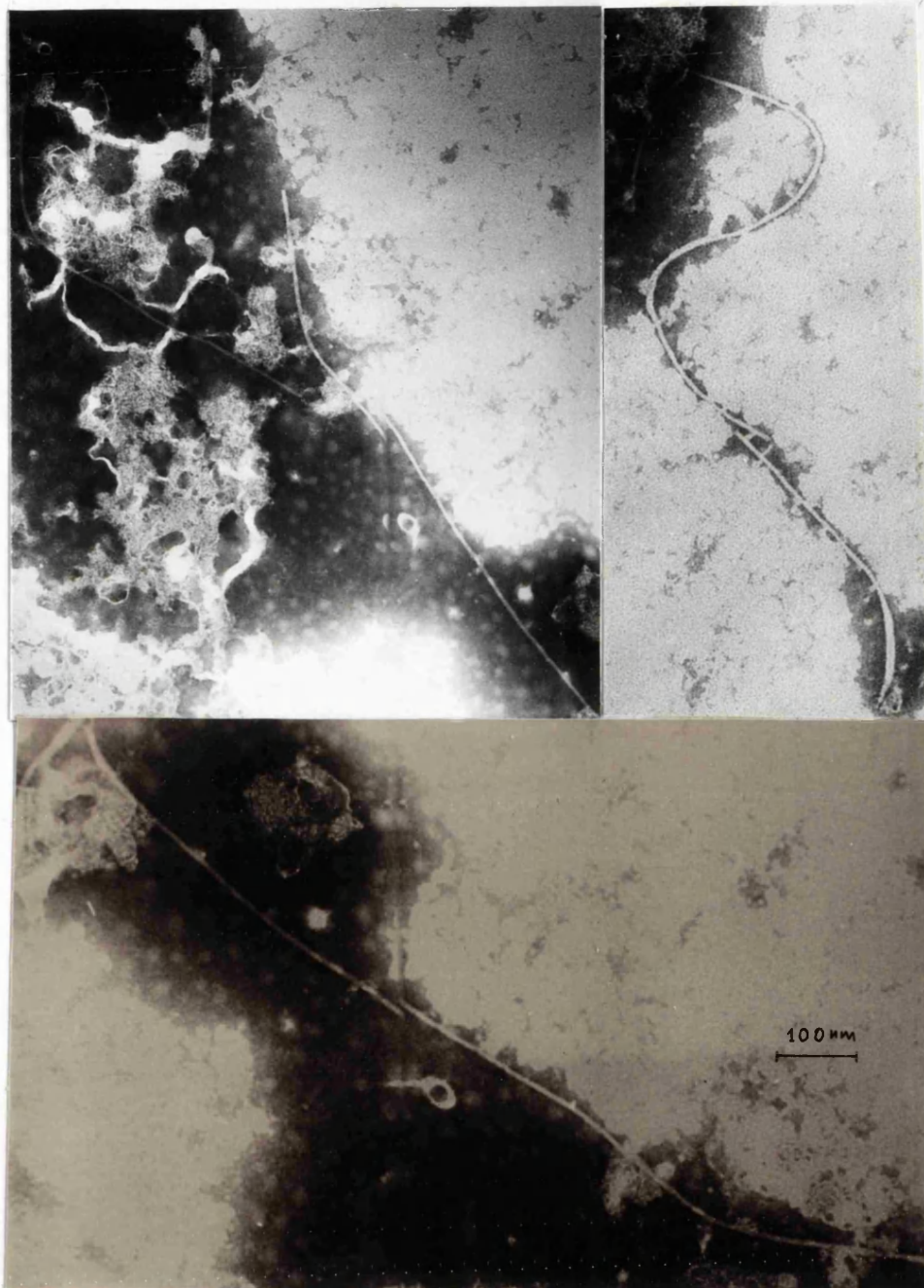
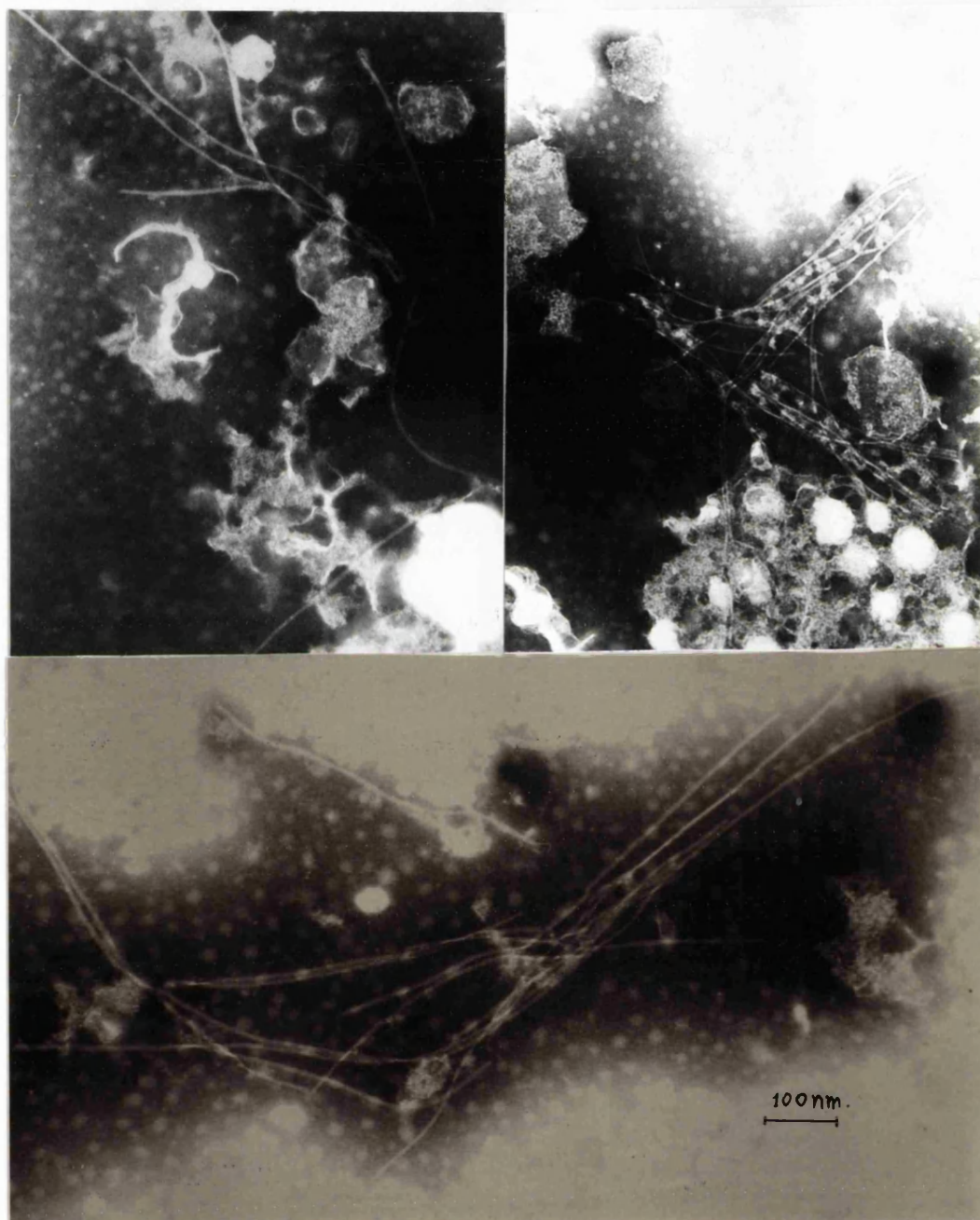


Plate 11 Electron micrograph of leaf squash
preparation of CV from *N. cleavelandii*
stained in 20 g/l PTA, pH 6.5
(b). "tangled" clump of particles.

(b)



3 were 2000-2168 nm. Particle width varied between 10-12 nm. depending on the stain. A central stain-penetrated canal was partially visible in several particles in uranyl formate and there was slight evidence of cross-banding. This was not seen in tungstophosphate.

Flexuous rods similar to these in *N. clevelandii* were seen in experimentally infected *C. quinoa*, *Pisum sativum*, *Phaseolus vulgaris* and *Cassia corymbosa*, both in the original shrub, and in seedlings mechanically inoculated with CV1 .

Ultrathin sections

Systemically infected *N. clevelandii* and *Cassia* leaves were prepared for thin sectioning with the procedures described by Karnovsky (1965) and Meck (1970). Portions of leaves showing strong mottle were chosen as the starting material. Strips about 3-8 mm. were cut from the leaf, the cut being made perpendicular to small veins near the leaf edge. The strips were fixed for 3 hr. in 0.1 M cacodylate buffer (Sabatini, 1963) containing 3% glutaraldehyde at pH 7.4 and transferred to 0.1 M cacodylate buffer at 4 °C. overnight. They were then cut down into a rectangular block size 1 x 5 mm. and postfixed for an hour in 2% osmium tetroxide (OsO₄) dissolved in 0.05 M buffer (cacodylate pH 7.4) and

washed two times for 10 min. each in 0.05 M buffer. Subsequently, the samples were put in 1% tannic acid in 0.1 M buffer at pH 7.4 for 1 hr. before being washed again in 0.05 M buffer(two times) for 10 min. The dehydration was performed in acetone serial dilution of 50, 70, and 100% respectively for 10 min. each. Tissues were embedded firstly in 1:1 (v/v) acetone resin mix and left on a collar cell, rotated overnight. The next day they were left on the collar cell one more night shortly after being transferred to 1:3 (v/v). After 24 hr., the samples were polymerized in a mould with 100% Epon Araldite in an oven at 60 °C. for 2 days. The blocks were cut transversely with a diamond knife. Prior to examination in the electron microscope, sections were stained first for 10 min. in 2% aqueous uranyl acetate solution, and then for 2 min. in an aqueous lead citrate solution.

There was no evidence of any inclusion body or particle aggregates in the infected material which were characteristic of any individual virus or group of viruses (Christie and Edwardson, 1977).

3.12 Attempt to eliminate CV1 from infected *Cassia*
corymbosa cuttings.

3.12 a) Thermotherapy

Four *Cassia* cuttings from the original infected shrub were incubated at 38°-40°C. in an illuminated, constant temperature growth cabinet (16 hr. photoperiod). After 6 weeks, plants were assayed for virus. Young *Cassia* leaves were harvested from one shoot on a plant from the top to the tenth node. After homogenising in a few drops of phosphate buffer, samples from each plant were inoculated to *C. quinoa* plants. Leaves were tested by this method every two weeks for 3 months.

Infectivity was still detected even from samples 3 months after heat treatment. Similar numbers of lesions (usually less than 12) were produced on *C. quinoa*, whichever node was sampled. Lesion numbers were also of the same order of magnitude after 3 months at 38°-40°C. as after 1 month. In addition, plants did not grow well at these temperatures, and by the end of the experiment some plants had begun to defoliate.

The failure to eliminate virus at the temperatures used may have been due to the ability of the virus through adaptation to replicate or

survive in a host grown normally in tropical/sub-tropical climates. Wang and Hu (1980) also reported that heat treatment was less efficient in inactivating rod and filamentous viruses than inactivating small spherical viruses.

3.12 b) Chemotherapy

Potential antiviral agents used for virus elimination were tested by the leaf discs method as a "prescreen", with the objective of testing promising chemicals further using explant culture. Most of the chemicals used for this experiment have been reported to inhibit viruses, for example, zinc and some metal ions(Korant et al., 1973), sodium dodecyl sulfate (SDS) (Longberg-Holm and Noble-Harvey, 1973), guanidine (Dawson, 1984), and amantadine-HCl(Horst and Cohen, 1980).

The experiment followed Dawson's method (1984). Concentrations of 0.01, 0.1, 1 and 10 mM of amantadine-HCl, guanidine, SDS, ZnCl₂, MnCl₂ and CaCl₂ were made in sterile distilled water. Ten to twenty discs (diameter c.7 mm.) per treatment were punched from systemically-infected *N. clevelandii* leaves by cork-borer No.3 . The discs were floated on the chemical solutions in 3.5 mm. Petri dishes and kept in a plant growth cabinet at 25°C. with a 14 hr. photoperiod of approximately

15000 lux. Control discs were floated on sterile distilled water. After 96 hr. of incubation, all discs were removed from the chemicals and frozen at -20°C. until infectivity was assayed on *C. quinoa* using a Latin square design.

The results are presented in Table 18.

Table 18 Effect of the chemicals on the multiplication of CV1 in leaf discs.

Chemical	Control	Mean no. lesion per half-leaf			
		Solution concentration (mM)			
		0.01	0.1	1.0	10
Guanidine	8	52	67	45	20
Amantadine-HCl	7	5	12	4	1
SDS	1	2	27	17	1
ZnCl ₂	1	0	4	4	1
MnCl ₂	1	13	2	1	1
CaCl ₂	1	3	9	1	0

None of any chemicals tested appeared to inhibit the multiplication of CV1 in leaf discs. On the other hand, guanidine, amantadine-HCl and SDS at 0.01, 0.1 and 1.0 mM stimulated virus replication compared with the control, particularly at 0.1 mM which produced the highest level of infectivity. At 10 mM virus infectivity usually declined compared to other concentrations possibly due to some phytotoxicity, although except for guanidine lesion

numbers were little different from those of the control treatment. The results were generally highly variable and except for guanidine treatment lesion numbers were low. So it is difficult to draw firm conclusions from these data.

3.13 In vitro culture of *Cassia corymbosa*

The culture was established using nodal segments (1.5-2 cm.in length) from young stems of the original infected *Cassia* plant in a heated glasshouse. After surface sterilization, explants were placed vertically on a nutrient medium (either WPM or MS medium containing 0.5 μ M BA). Cultures were kept in a growth room at 24°-25°C. and light intensity at 30000 lux. for 16 hours a day.

After 14 days of establishment in vitro, over 75% of nodal explants were found to be visibly free of contamination. Within 3 weeks nodal cuttings taken from young stems and grown in vitro produced shoots of greater length when grown on WPM rather than MS based medium. This result was similar to that of Cheng (1978) who studied a range of media for woody plants and found that establishment and multiplication were better in media with a less salt concentration than MS. The leaves from WPM media were bigger both in length and

width although the number of nodes were the same (plate 12). All leaves in both treatments, however, showed clear mottling and slight twisting at the tip. After the third subculture to fresh medium, however, the leaves started to turn yellow at the base and fell off leaving the explant leafless. It was not clear, whether this indicated that the media was unsuitable for growth of *Cassia in vitro* beyond culture initiation, or whether virus was causing the growth in vitro to stop.

3.14 Discussion

The virus isolate from *Cassia corymbosa* could be distinguished from other *Cassia* viruses that have already been reported either by its particle shape and size, or by its host reaction. For example, this CV1 had long flexuous particles with highly variable lengths which ranged from 150 nm to 2168 nm., while Verma and Niazi (1974) described a potexvirus which infected *C. occidentalis* and had flexuous particles of 550-580 nm. length. Another two reports of flexuous rod-shaped particles in *Cassia spp.* were the potyvirus tobacco etch virus (Debrot, 1976), and another virus belonging to potyvirus group, isolated from *C. hoffmannsegii* by Paguio and Kitajima (1980). The carlavirus isolated by Lin et al. (1979) from *C.*

Plate 12 Growth comparison of *Cassia* explants in
tissue culture (left) on M.S. (right) on
W.P.M media.



sylvestris in Brazil had particles of 640 x 15 nm. In contrast to CV1, their isolate failed to infect *Chenopodium spp.*, and leguminous genera other than *Cassia*.

The tendency of CV1 particles to fragment, even in leaf dips and squashes, and the absence of any clear modal length, made it difficult to determine accurately the size of the intact particle. A small but probably significant proportion of particles, however, were over 1000nm. in length suggestive of a subgroup I closterovirus affiliation (Lister and Bar-Joseph, 1981). On the basis of the in vitro properties (CV1 was moderately unstable) and electron microscopy CV1 could tentatively be assigned to the closterovirus group.

The relative molecular mass estimates for the presumed replicative form of the *Cassia* virus genome were similar to that reported for the closterovirus, citrus tristeza (Dodds and Bar-Joseph, 1983) which has particles of about 2000 nm. length. The major top band of dsRNA with a high Mr. of $13-15 \times 10^6$ was difficult to detect when host DNA was also present, as those two bands comigrated on the gel. After DNAase digestion, however, the dsRNA band was clearly visible. Because of poor spatial resolution of high Mr markers, the

estimated Mr. of CV1 dsRNA should be confirmed using an alternative method, for example, by contour length measurements in electron microscope (Bruenn, 1980). The Mr. of CV1 dsRNA was equivalent to a genera ssRNA with Mr. about 7×10^6 .

The dsRNAs recovered from extracts of infected bean produced two bands of Mr 13.0×10^6 and 1.7×10^6 unlike the extract from *N. clevelandii* where only one dsRNA species with an estimated Mr. of 13.0×10^6 was detected. It was interesting that dsRNA extracted from leaves of the original *Cassia* plant with clear symptoms, resulted in three major bands, whereas, only bands 1 and 3 were visible from plants artificially inoculated with CV1. Presumably, the missing band was either in too low a concentration to be visible on the gel, or was absent. Dodds and Bar-Joseph (1982) found that dsRNA patterns of beet yellow virus (BYV) in two different hosts, *Claytonia perfoliata* and *Beta vulgaris*, were not the same. It is possible that the seedlings *C. corymbosa* had a slightly different genome than the naturally infected shrub and thus induced slightly different dsRNA patterns. On the other hand, Valverde et al (1985) reported that in their studies with several elongate viruses, host effect was only on yield of dsRNA on each sample, not the pattern. In the present case,

hybridisation studies will be needed to determine if the additional band in the parent shrub was a subgenomic component of CV1 or indicated infection by a distinct virus-like agent. The estimated Mr. of the dsRNA 2 was however too low for CV2 described in part B of this Chapter which was also isolated from this shrub.

Thin sections of infected leaf tissue revealed no characteristic inclusions indicative of the closterovirus or any other group. No particles were seen in phloem cells, where according to (Bar-Joseph and Murrant, 1982) closteroviruses induce swelling and disintegration of chloroplasts and mitochondria, and accumulation of osmiophilic globules in the mitochondria, and of phytoferritin in the chloroplast (Bar-Joseph and Murrant, 1982). More detailed studies with healthy *Cassia* material for comparison will be needed if more subtle structural changes are to be detected.

To achieve purification, there are many critical factors which have to be considered, if the viruses are to be used as antigens for use in serological tests and in the preparation of antisera. The problems involved in this study were, firstly, the virus was unstable particularly during homogenisation, and susceptible to organic solvents

during clarification. Secondly, the test plants selected for purification yielded relatively low levels of virus as determined by infectivity assay. Possibly this was due to the adsorption of viruses by the particulate fraction of plant cells after they were disrupted (Lister and Bar-Joseph, 1981). Restriction of CV1 to phloem cells, a problem with some closteroviruses (Price, 1966; Bar-Joseph and Murrant, 1982) was not a likely explanation as CV1 was readily sap transmitted. Thirdly, experiments on resuspending conditions showed that the infectivity of concentrated partially-purified extracts initially increased with dilution, suggesting either the presence of inhibitors in the residual impurities, or that virus tended to aggregate or adhere to these impurities unless solutions were diluted. Since aggregation is a major problem in purifying elongated viruses (de Sequeira and Lister, 1969) this is the more likely explanation. Further studies are needed to investigate suitable host plants for virus multiplication to develop a sufficiently "gentle" purification method to preserve particle integrity.

Purification will be needed before CV1 can be properly characterised chemically to confirm its closterovirus group affinity, and to

establish its relationships, if any, to other viruses.

Cassia corymbosa seedlings inoculated with a single lesion isolate of CV1 produced symptoms typical of the original disease. The reisolation of a virus with an test plant reaction and particle morphology to the original isolate comes close to satisfying "Koch's Postulates". In addition, similar dsRNA profiles on gels loaded with samples from the original shrub and the inoculated seedling, suggested the same virus was involved in both diseases. The name cassia severe mosaic virus has provisionally been given to the isolate causally associated with the disease in the shrub (Ngamyeesoon and Hicks, 1989).

PART B : CASSIA VIRUS ISOLATE NO.2

During routine tests for CV1 from the *Cassia* shrub, atypical symptoms were infrequently observed on *C. quinoa* assay plants. Chlorotic local lesions, which are the typical response to CV1, developed on inoculated leaves, but uninoculated leaves produced a systemic mottling different from the occasional systemic chlorotic lines and spots which occurred in summer on plants infected with CV1. Accordingly, these systemic leaves were back tested to *C. quinoa* seedlings. After 10 days, inoculated leaves developed yellow green chlorotic spots, some ringspot lesions, and a systemic chlorotic mottle symptom. Three successive single lesion cultures from *Chenopodium amaranticolor* were established and the final isolate was designated as CV2.

3.15 Herbaceous host range

Virus was transmitted from systemically-infected *C. quinoa* leaves using 0.1 M phosphate buffer pH 7 to a wide range of herbaceous plants. Symptoms on test plants are given in Table 19.

**Table 19 Symptoms induced on herbaceous hosts
by CV2**

<u>Host Plants</u>	<u>Symptom expression</u>
<u>Chenopodium album</u>	NL/C,N,D
<u>C. amaranticolor</u>	CL/C,M,D→St
<u>C. foetidum</u>	CL/NT,Dth
<u>C. murale</u>	CL,RS/NT Dth
<u>C. quinoa</u>	CL/M,D St
<u>Cucumis sativum</u> cv. "Parisienne Pickling"	CL,RS/CS,CM→St
<u>Nicotiana clevelandii</u>	SI/LP,RS
<u>N. debneyi</u>	SI/CM,VY,D
<u>N. megalosiphon</u>	SI/VY,NF,D
<u>N. rustica</u>	SI/CR,M
<u>N. tabacum</u> "Xanthi"	SI/CM
<u>N. tabacum</u> "White Burley"	SI/CM,NS,VN
<u>Phaseolus vulgaris</u> cv."The Prince"	SI/CM,NT→Dth

Abbreviations : local reactions/systemic symptoms

C = chlorotic or chlorosis	LP = line pattern
N = necrotic or necrosis	VY = vein yellowing
L = local lesions	VN = vein necrosis
M = mottle	D = distortion
F = flecking	St = stunting
R = rings	Dth= death
SI= symptomless infection	RS = ring spots
Y = yellowing	NT = necrosis tips

The symptoms on *Chenopodium spp.* (plate 13) consisted of indistinct local lesions, appearing within 5 to 7 days , with a systemic chlorotic mottle after 10 days, followed by distortion and stunting with apical necrosis.

In *C. murale* and *C. foetidum* local lesions were usually followed by collapse and death of the plant within 3-4 weeks (plate 14).

Nicotiana spp. produced symptomless local infection, followed by a systemic chlorotic mottle, rings and lines (plate 15). In summer symptoms tended to disappear.

Phaseolus vulgaris :Within 10-14 days, inoculated leaves showed brown necrotic lesions followed by a systemic necrosis and distortion of young leaves, and in winter a die-back from the shoot tip (plate 16).

Since there was no reliable local lesion host for CV2 , *C. quinoa* was used in a systemic reaction assay (10 plants per treatment). This plant was also a good source of virus for determing physical properties and purification.

Plate 13 Systemic chlorotic mottle on *C. quinoa* (a) and apical necrosis on *C. album* (b) infected with CV2.

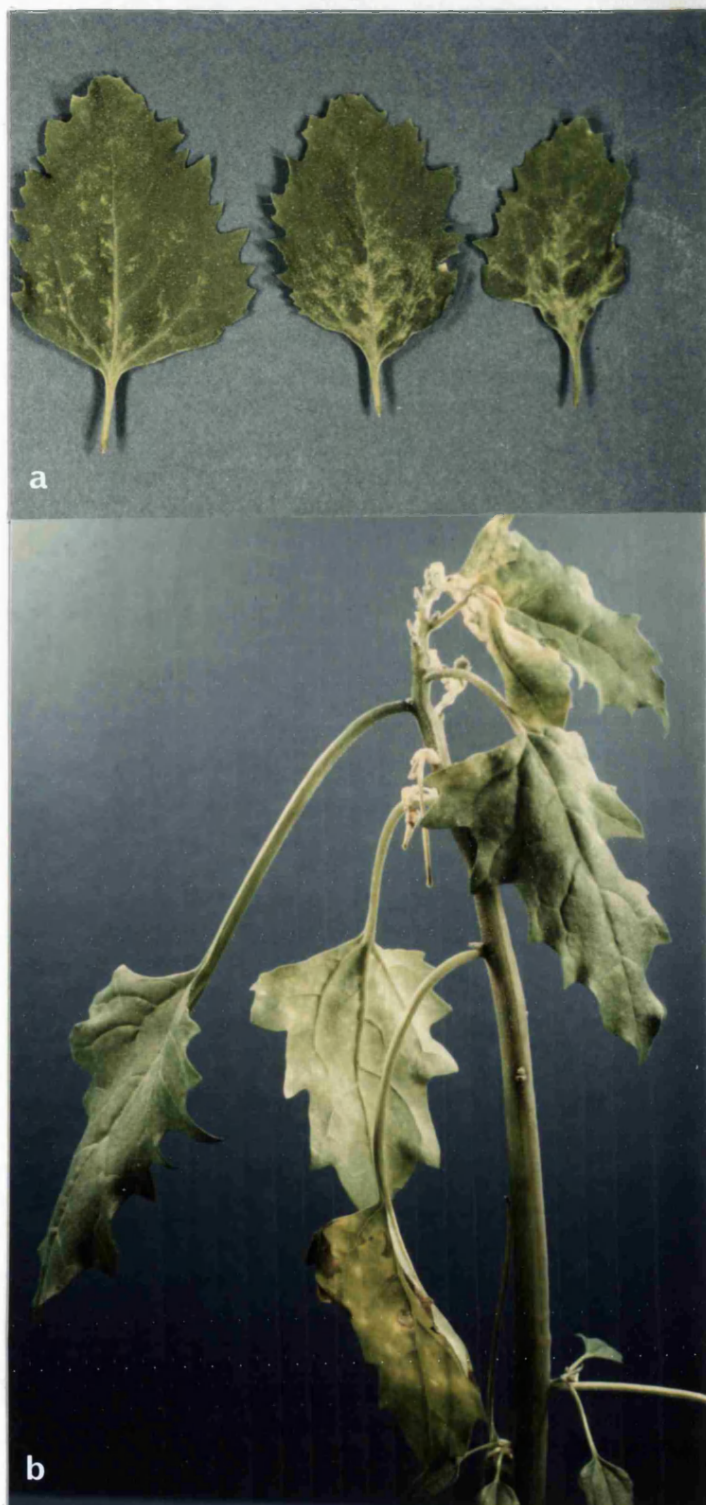


Plate 14 *Chenopodium murale* (left) *C. foetidum* (right) mechanically inoculated with CV2 showing collapse and death of the plants.



Plate 15 Systemic symptom on *Nicotiana clevelandii* infected with CV2.



Plate 16 Distortion of young leaves and shoot tip die back on *Phaseolus vulgaris* infected with CV2



3.16 In vitro properties of CV2

Sap for these tests was prepared from systemically-infected leaves of *C. quinoa* in phosphate buffer, (see Section 2.09). Samples were assayed for infectivity on *C. quinoa* seedlings (10 replicates).

3.16 a). Dilution end-point

The DEP of CV2 was 10^{-3} to 10^{-4} in two determinations.

3.16 b). Thermal inactivation point

The TIP of the isolate was 50°-60°C.

3.16 c). Longevity in vitro and storage

The isolate retained infectivity for 32 but not 64 days at room temperature.

Chopped leaves of *C. quinoa*, infected with CV2 stored dry over anhydrous calcium chloride and silica gel at -20 °C., retained infectivity for at least 14 months (the longest period tested).

3.17 Seed transmission of *Cassia* virus isolate 2

To obtain further information on CV2 which might be of diagnostic value, seed transmission was studied.

Seeds collected from an infected *C. quinoa* were sown in 8.5 x 6 inch size trays containing Levington Universal Compost and germinated under mist. In a sample of 100 progeny seedlings assessed for infection visually, about 75% of *C. quinoa* seedlings showed mottling on primary leaves. These seedlings were backtested onto healthy *C. quinoa*. All were found to be infected.

3.18 Double-stranded RNA analysis

The extraction was performed using 20 g. of infected *C. quinoa* leaves (Section 2.14). The electrophoresis was performed on 6% polyacrylamide gels at 5 mA/tube for 4 hr. The sample was run in parallel with dsRNA extracted from TMV-infected *N. tabacum* "White Burley" and CMV - infected *N. tabacum* "Xanthi" to provide standards for relative molecular mass estimation.

After DNAase digestion nucleic acid extracts from *C. quinoa* (CV2) produced two major and two minor bands (in term of concentration) on polyacrylamide gels. Using the above relative

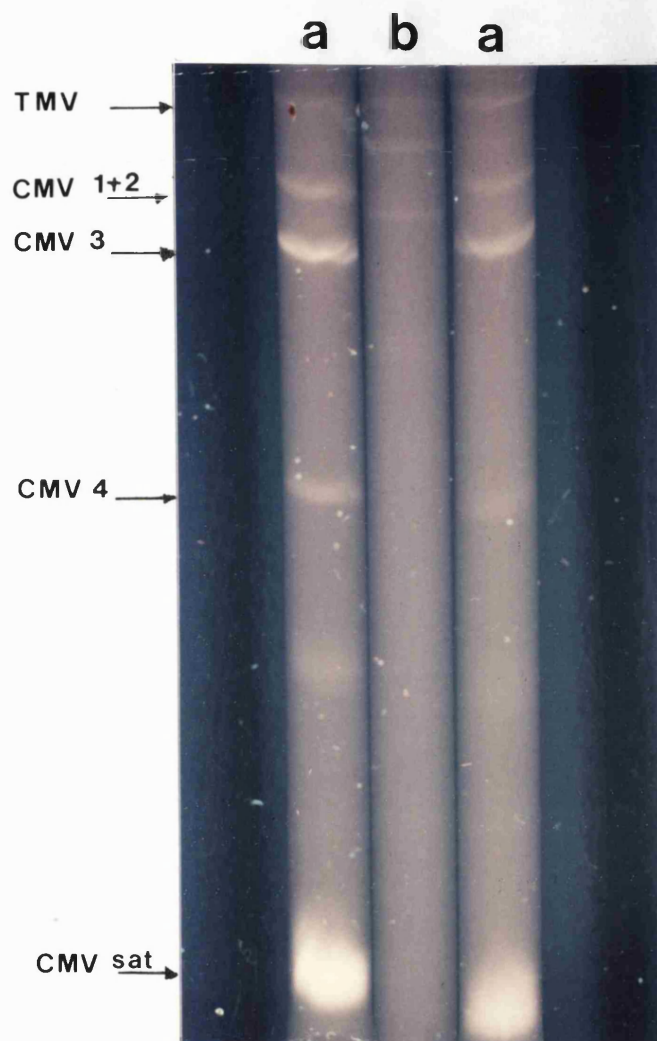
molecular mass markers, estimates for the 4 dsRNAs were 4.0×10^6 , 3.4×10^6 , 1.6×10^6 and 1.10×10^6 (plate 17).

3.19 Serology

Preliminary observations on general host reaction, and the presence of high levels of seed transmission in *C. quinoa* suggested CV2 could belong to the nepovirus group (Murant, 1981a). Other data (dsRNA analysis, in vitro properties) were also consistent with this diagnosis. To confirm this identification, antisera to three nepoviruses were used in gel double diffusion tests, with CV2 antigen.

For these tests, crude sap from systemically-infected leaves of *C. quinoa* or *C. amaranticolor* were used (undiluted) as sources of CV2 antigen, and were reacted with antisera to arabis mosaic virus (ArMV), strawberry latent ringspot virus (SLRV) or raspberry ringspot virus (RRV). After overnight incubation a reaction line had formed between antigen wells and antiserum to ArMV (*Daphne* isolate) used at dilution of 1:4 (v/v). No precipitin lines developed with healthy sap or normal serum controls, or with antisera to RRV and SLRV.

Plate 17 Ethidium bromide stained polyacrylamide gels showing dsRNA from (a) samples of TMV or CMV-infected tobacco or (b) CV2 infected *C. quinoa*. (arrows indicates dsRNA)



3.20 Bait-testing for viruliferous nematodes

Because CV2 was serologically identified as ArMV an experiment was studied to determine if CV2 was present in the soil under the *Cassia corymbosa* shrub.

Soil samples about 6-9 inch-deep from around the rhizosphere of the naturally-infected *Cassia* shrub were taken for bait-testing. Seedlings of 4 *Chenopodium quinoa* and 4 *C. amaranticolor* were transplanted into 9 cm. pots containing soil samples; cucumber seeds were sown directly into soil in pots. No symptoms developed on *Chenopodium* bait plants within 4 weeks of transplantation, nor on cucumber seedlings within 4 weeks of germination. Samples of root, stem and leaf tissue from each of the baits plants were assayed on *C. quinoa*, but no infection was detected.

These results indicated either low populations of viruliferous vector nematodes in soil around the shrub, or that the plant was already infected with ArMV before it was planted at Bath.

3.21 Partial purification of CV2

Limited attempts at purification were made using systemically-infected leaves of *C. quinoa* as a source of virus. The purification

protocol incorporated clarification by butanol-chloroform (Steere, 1956) and PEG precipitation (Gooding & Hebert, 1967).

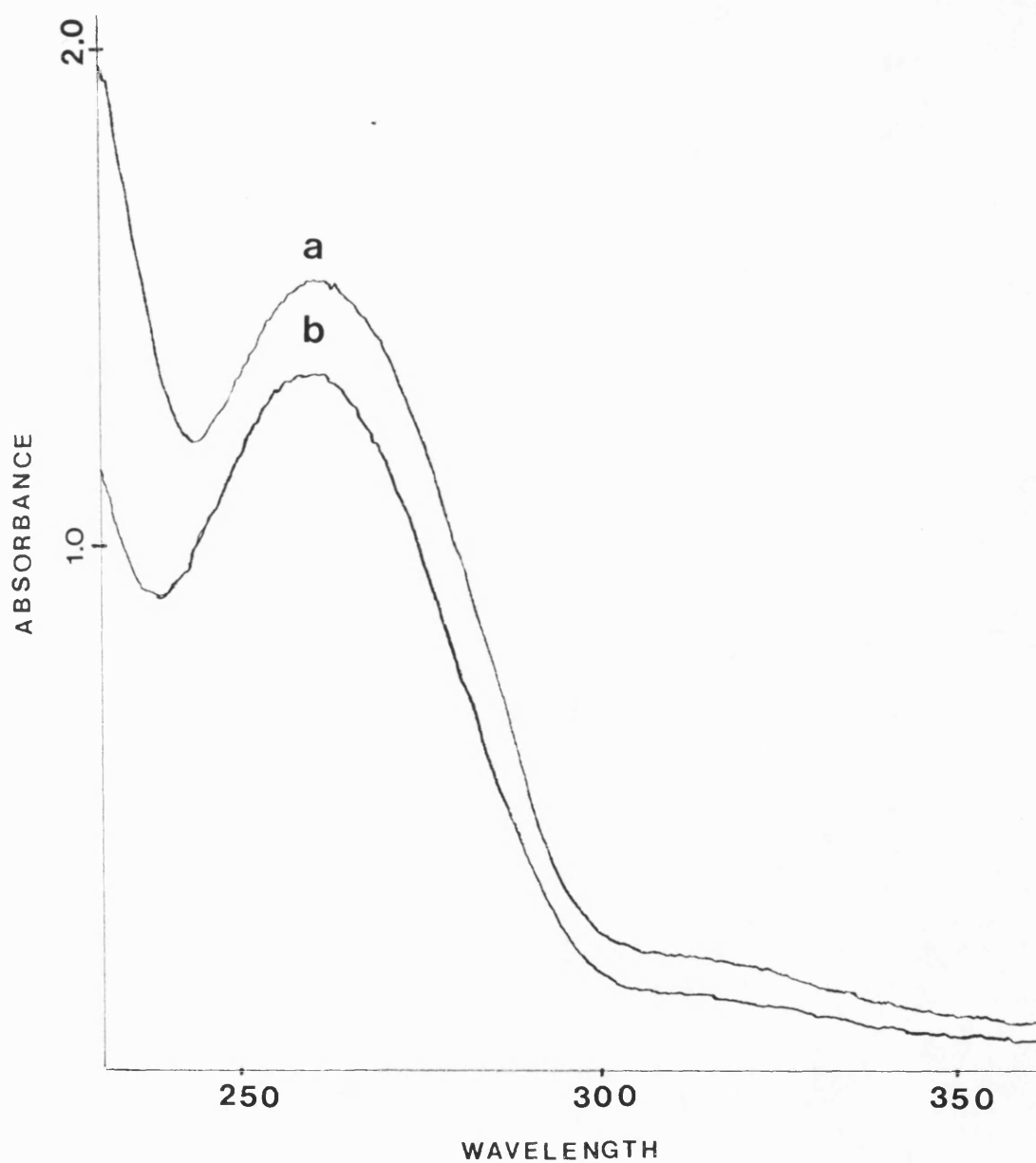
Leaf samples of 50 g. were homogenised in a Wareing blender with 100 ml of cold 0.05M phosphate buffer, pH 7.5 and filtered through muslin. The filtrate was stirred at 4°C. and 1 vol. of a 1:1 (v/v) mixture of n-butanol and chloroform added slowly. The emulsion was stirred for a further 30 min. and then broken by centrifugation for 30 min. at 8000 g to pellet denatured host material. The clear supernatant, containing virus, was collected and divided into two equal volumes. Polyethylene glycol Mr of 20,000 or 8000, was added to each supernatant at 10% (w/v) and 0.3 M NaCl the solution adjusted. The mixtures were stirred at 4 °C. for 2 hr. before the clarified preparations were centrifuged for 30 min. at 12,000 g. The pellets, containing virus, were resuspended overnight at 4°C. in a total of 3 ml of buffer. Resuspended pellets were clarified by centrifugation for 15 min. at 4000 g, tested for infectivity on *C. quinoa* and the U.V. absorption spectra determined.

After final clarification undiluted samples were assayed for infectivity which was found to be higher for preparations made with PEG 8000 (DEP 10^{-2}) than PEG 20,000 (DEP 10^{-1}).

The absorption spectrum of a 1:100 (v/v) dilution of each partially purified preparation was determined (Figure 5). Typical nucleoprotein absorption spectra were obtained with both samples. However, the uncorrected A 260/280 ratios were 1.86 and 1.56 for samples precipitated with PEG Mr 8000 and 20000, respectively. The 260/280 ratio for isometric particles with an RNA content of 30% is reported to be about 1.73 (Gibbs and Harrison, 1976). A lower A260/280 ratio of sample precipitated with PEG Mr 20,000 suggested contamination with extraneous protein. Therefore, in future work 10% PEG mol.wt. of 8000 was used to concentrate the virus.

The role of metal ions in the preservation of infectivity or structural integrity is well established for some viruses (Lister and Hadidi, 1971; Fulton, 1971). For other viruses, metal ions reduced infectivity by causing aggregation. Indirect evidence for a role of metal ions can be obtained from the effects of chelating agents such as EDTA on virus stability (Matthews,

Figure 5 Absorption spectra of partially-purified preparations of CV2 concentrated by
(a). 10% PEG Mr 8,000
(b). 10% PEG Mr 20,000



1981). It was considered worthwhile, therefore, to investigate CV2 purification with and without 0.01M EDTA.

The purification method was done as described above using 50 g of systemically *C. quinoa* leaves for each treatment. The viruses were precipitated by PEG Mr 8000 and 0.3 M NaCl. The pellets were resuspended in 3 ml of 0.05M phosphate buffer pH 7.5 overnight. When samples were assayed for infectivity (DEP) in *C. quinoa*. Differences in end-point were detected. For preparations in EDTA, the DEP was 10^{-3} to 10^{-4} , without EDTA, 10^{-1} to 10^{-2} . Moreover, the absorption spectra (Figure 6) gave uncorrected A₂₆₀/280 ratios of 1.7 and 1.5 for samples with and without 0.01 M EDTA, respectively.

In a further experiment, equivalent samples purified as above, with or without EDTA, were subject to one cycle of CPG chromatography equilibrated in, respectively, buffer with 0.01 M EDTA or plain buffer both at pH 7.5. When the column outflow was monitored at 254 nm. the virus peak (determined serologically) from the sample with EDTA was markedly higher than the sample without EDTA (Figure 7). Spectral analysis of the fractions from major peak from each treatment gave a A₂₆₀/280 ratio

Figure 6 Absorption spectra of partially-purified preparations of CV2 extracted in 0.05 M phosphate buffer with (a) 0.01 M EDTA and (b) without EDTA

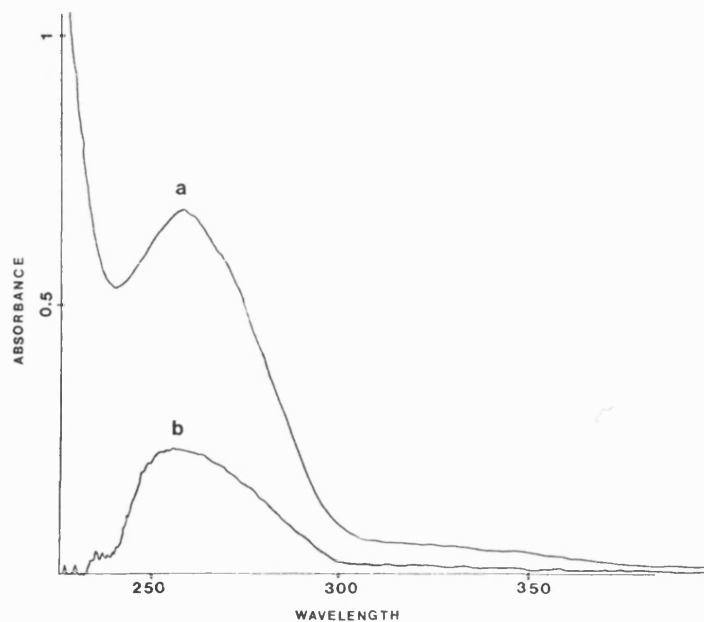
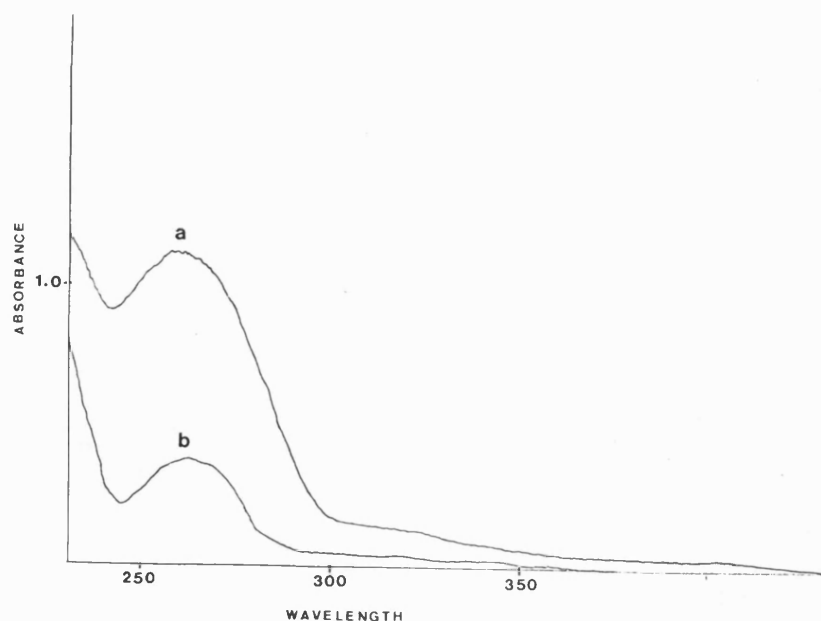


Figure 7 Absorption spectra of partially-purified preparations of CV2 (after passage through CPG column) in 0.05 M phosphate buffer with (a) 0.01 M EDTA (b) without EDTA.



of 1.6 and 1.45 for buffer with EDTA, and buffer without EDTA treatment, respectively. Neither of the ratios departed significantly from that expected for nepoviruses (Murant, 1981). Using an assumed extinction coefficient of 7 for nepoviruses (Murant, 1981), the concentration of sample with EDTA was 3.3 mg/ml, while sample without EDTA was 1.2mg/ml. Infectivity assay of samples from the column showed both preparations (with and without EDTA) to be infective, although detailed comparisons were not made.

3.22 Electron microscope

Observations of partially-purified samples (EDTA purified, Section 3.21) in the electron microscope stained with 2% potassium phosphotungstate (PTA) showed clusters of isometric particles about 28-30 nm in size. Electron micrographs showed some particles completely, some partially, and some unpenetrated by negative stain (plate 18 a). These particles probably correspond to three components, apparently empty protein shells (T) and two kinds of nucleoprotein (M and B) which are present in preparations of ArMV and some other nepoviruses. (Lister and Murant, 1967; Murant, 1981).

Examination of a leaf squash preparation from systemically-infected leaves of infected French Bean (*Phaseolus vulgaris*) showed moderate concentrations of isometric virus like particles, some with hexagonal outlines (plate 18 b) about 30 nm. diameter (in 2% PTA). Tubules seen with some nepovirus infections (Murant, 1981 a) were not observed. Harrison and Nixon (1960) reported isometric particles, about 30 nm in diameter with 5 or 6 sided angular outline for several nepoviruses including ArMV (Murant, 1981 a).

3.23 Relative molecular mass of capsid protein of CV2

Partially- purified virus samples in 0.05 M sodium phosphate buffer pH 7, containing 1% SDS and 1% 2-mercaptoethanol were dissociated by boiling for 5 min. A few grains of bromophenol blue and 100 μ l/ml glycerol were added to the virus samples, and also to 3 protein standards : glyceraldehyde phosphate dehydrogenase, β -lactoglobulin and serum albumin. Samples of about 100 μ l were loaded separately onto each gel. Electrophoresis was performed for 4 hr. at 8 mA/gel (see Section 2.16).

After electrophoresis and staining in Coomassie blue a major polypeptide with Mr 52,000 was detected (plate 19).

Plate 18 Electron micrograph of CV2 stained in 2% PTA pH 6.5.
(a) Partially purified preparation extracted in 0.05 M phosphate buffer with 0.01 M EDTA

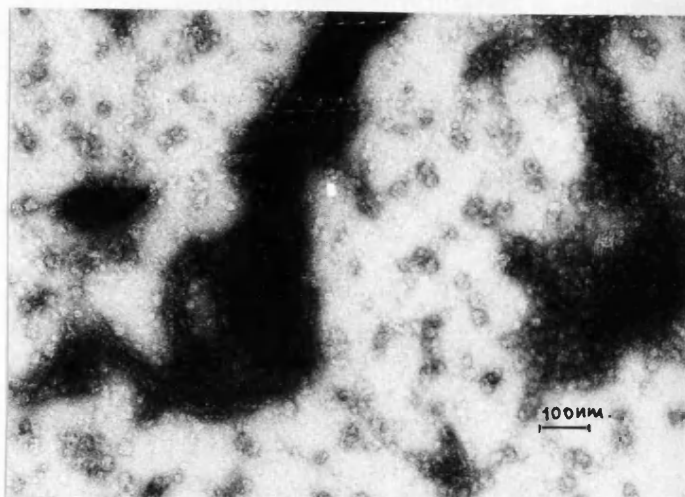


Plate 18 (b) Leaf squash of infected *Phaseolus vulgaris* with CV2.

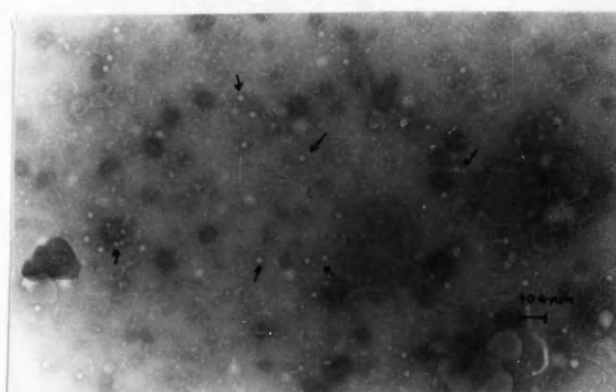
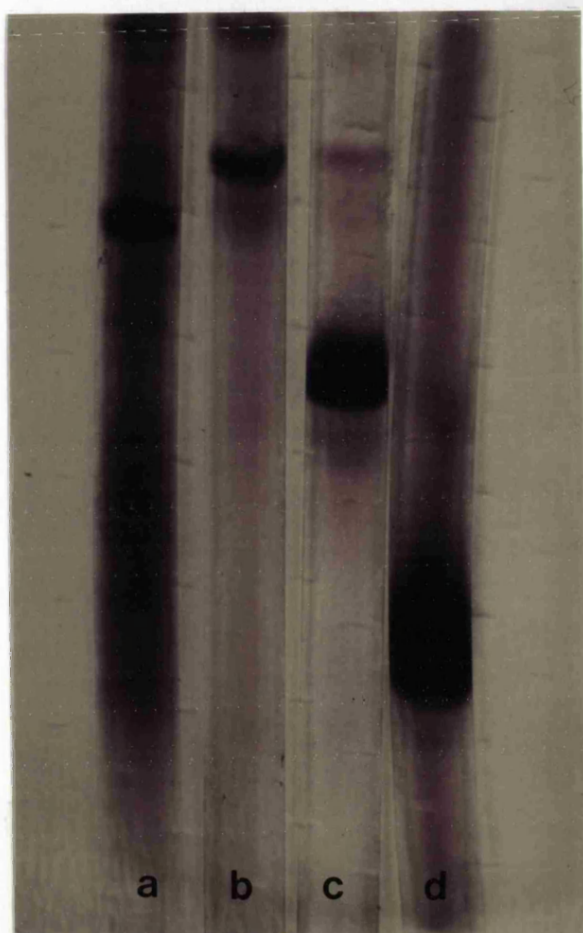


Plate 19 Determination of CV2 coat-protein Mr by SDS
polyacrylamide gel electrophoresis.
Gels from left to right (a) protein CV2
(b) serum albumin (68000) (c) GPD (36000)
(d) β -lactoglobulin (18400)



3.24 Return inoculation of CV2 to *Cassia corymbosa*

In the present study, attempts were made to reproduce symptoms in seedling *Cassia corymbosa* by inoculation with CV2. Six seedlings about 6 weeks old were mechanically inoculated with partially-purified virus preparations after 2-3 days in the dark. After inoculation plants were kept in a glasshouse at 15°-35°C. for symptom observation.

No symptoms were observed on inoculated or non-inoculated leaves after 12 months. No infection was detected in inoculated plants when leaves were backtested to *C. quinoa* seedlings.

3.25 Discussion

Several lines of evidence suggested that CV2 was one of the nepoviruses. These included, a wide host range, and the production of ringspots on several hosts. Although not a major feature of host reaction to CV2, there was also a tendency for symptoms to fade in some hosts (e.g. *Nicotiana*) particularly in summer. Murant (1981a) considered recovery to be an important diagnostic characteristic of nepoviruses, although the same phenomenon is also seen with some cucumoviruses (Kaper and Waterworth, 1981). Further evidence that CV2 was a nepovirus came from the high levels of

seed transmission detected in *Chenopodium quinoa* . Of the viruses with isometric particles, confirmed for CV2 by electron microscopy, seed transmission is a general characteristic of the nepoviruses (Matthews, 1981).

Additional evidence for nepovirus affiliations were the presence of a major protein component with a Mr of 52,000. This value was slightly lower than that given for most nepoviruses which have values of 54,000. It was, however, higher than that of any other isometric virus of comparable size and, thus, was a useful diagnostic feature. The major dsRNA species of CV2 migrated at a similar rate to the dsRNA of tobacco mosaic virus. This was slightly lower than that expected for most nepoviruses. The second dsRNA species of CV2 was within the range expected for the dsRNA of nepovirus RNA 2. A third band of dsRNA and a very faint fourth band possibly a satellite dsRNA, were also detected. Several nepoviruses including ArMV have low Mr satellite molecules about $0.4-0.5 \times 10^6$ associated with them (Murant and Mayo, 1982).

It was not possible from host reaction alone to identify CV2 beyond the probable nepovirus grouping. The systemic infection in

Chenopodium amaranticolor , however, indicated that it was not raspberry ringspot virus (Murant, 1981). Serological tests confirmed CV2 as an isolate of ArMV. Symptoms on herbaceous hosts were generally typical of ArMV (Murant, 1970; Murant, 1981 b) although symptom differences were noted, for example, in tobacco, between CV2 and ArMV isolates from other woody hosts (Hicks, 1979; Perkins, 1987).

The presence of a second virus in the glasshouse- grown *Cassia corymbosa* could not be predicted from the studies on CV1. Analysis of dsRNA extracted from the naturally-infected shrub did not show bands which corresponded to ArMV dsRNA (Section 3.10). This, together with the erratic occurrence of CV2 in CV1 inocula, indicated that CV2 was not uniformly distributed in the shrub.

All of the symptoms described on the original shrub could be accounted for by CV1 suggesting that CV2 produced few, if any, symptoms. Many nepoviruses, including ArMV (Murant, 1981a) often occur as symptomless or mild infections particularly in woody perennials, such as hedgerow plants (Harrison and Winslow, 1961). Furthermore, if infection is soil-borne there may be a period of at least one year before symptoms or virus can be detected in the foliage (Murant, 1981 b). In bait

tests, however, no evidence for the soil
transmission of CV2 could be found.

CHAPTER 4 : VIRUS INFECTION OF CARYOPTERIS CLANDONENSIS.

The genus *Caryopteris* is an eastern Asian genus of 15 species of deciduous, small shrubs, subshrubs and herbaceous plants. The flowers are blue or violet-blue often in axillary clusters, while leaves are short-stalked, toothed or toothless. The blooms have bell-shaped, deeply-five-lobed calyxes.

Caryopteris clandonensis is a hybrid species, the parents of which are *C. incana* and *C. mongholica*. From 2-4 feet tall, the majority (of the genus) are soft wooded shrubs. Several have been given varietal names such as "Blue Mist", "Heavenly Blue" and "Arthur Simmonds".

Schmelzer (1962-63) was apparently the first to report virus infection of *Caryopteris spp.* A sap transmissible virus was isolated from *C. incana* with yellow spots, which on the basis of in vitro properties, aphid and dodder transmissibility and host reaction, was later identified as a strain of alfalfa mosaic virus (Schmelzer, 1968); this virus was confirmed by cross protection and serology tests, by the same author. Schmelzer(1962-63) also isolated a strain of tomato black ring virus from

symptomless plants which produced a strong necrotic symptom on cucumbers. Neither virus seemed to have been re-introduced into healthy *C. incana*.

Schimanski et al (1977) detected cucumber mosaic, tomato black ring spot and arabis mosaic virus in *C. clandonensis* during a survey of virus infection on ornamental woody plants in the GDR. Schimanski et al . (1977) demonstrated an aphid-transmissible virus in both *C. incana* and *C. clandonensis* at a maximum of 2.6%. In both species cucumber mosaic virus was confirmed.

In a survey for viruses that infected woody ornamental plants at Long Ashton Research Station (Section A) two clones of *C. clandonensis*, out of nearly 30 samples were found with distinct virus-like symptoms, which later were associated with two viruses, designated CCLV1 and CCLV2.

PART A : VIRUS INFECTED CARYOPTERIS CLANDONENSIS
CLONE 7

4.01 Symptoms of infection and isolation of CCLV1

The infected plants of *Caryopteris clandonensis* clone 7 produced yellow blotching or chlorotic spots and rings on young leaves (plate 20). At the time of detection, the diseased

Plate 20 Yellow blotching on young leaves of
Caryopteris clandonensis clone 7
naturally infected with CCLV1.



plants did not show any obvious effects on growth or flowering, compared with adjacent apparently virus-free plants of the same clone.

A virus isolate was obtained by homogenising young *Caryopteris* leaves with clear symptoms in 0.05 M phosphate buffer pH 7.5 (1:3 w/v) and inoculating the extract to a range of herbaceous test plants. Ten days after inoculation, most of the *Chenopodium spp.* showed local lesions on inoculated leaves. The infectivity, however, was less than 10 lesions per leaf. Only two species of tobacco were used in this test; *Nicotiana tabacum* "White Burley" and "Xanthi". Faint yellow chlorotic spots appeared on inoculated leaves of both inoculated plant species, followed by mosaic symptom on systemic leaves. Other species failed to develop symptoms.

The virus from *C. clandonensis* was difficult to isolate throughout the year. An experiment was done, therefore, to see if the poor transmission was due to the presence of an inhibitor present in normal *Caryopteris* sap. Young leaves of apparently healthy *C. clandonensis* were homogenised in 0.1 M phosphate buffer 1:1 (w/v) pH 7.5 and the extract filtered through muslin before a dilution series of 1:10, 1:50, 1:500 and 1:5000 (v/v) was

made, in the same buffer; sap samples were stored in crushed ice until use. Sap from leaves of CCLV1-infected *N. tabacum* "Xanthi" was prepared in the same way, with a dilution series of 1:10 (w/v), 1:50 to 1:5000 (v/v). Each dilution of *Caryopteris* sap was tested for inhibitory activity by mixing with an equal volume of infected tobacco sap, leaving on crushed ice for 30 min and then inoculating the sap to *Chenopodium amaranticolor* in a 4 x 4 Latin square design. An equal weight of celite (4mg/ml) was added to each sample before inoculation. Tobacco sap (1:10) infected with CCLV1 diluted with an equal volume of plain buffer was used as the control treatment.

Table 20 Influence of *Caryopteris* sap on infectivity of CCLV1

<u>Dilution of tobacco sap with CCLV1</u>	<u>Mean no. lesions per leaf</u>			
	<u>Dilution of <i>Caryopteris</i> sap</u> <u>1:10</u>	<u>1:50</u>	<u>1:500</u>	<u>1:5000</u>
Control (1:10)	32	16	5	1
1 : 10	27	19	2	3
1 : 50	24	21	4	5
1 : 500	16	9	4	2

The result showed no great inhibitory effect of any sap virus combination, as infectivity decreased when the dilution of the *Caryopteris* sap increased. This suggested that

difficulties with isolation were associated with low virus concentrations in the leaf tissue, rather than the presence of inhibitors.

4.02 Host range of CCLV1

The culture of CCLV1 -infected *N. tabacum* "Xanthi" which came from three successive single lesion isolates in *C. quinoa*, was used as a source of inoculum for this study. Systemically infected leaves were ground in 0.1 M phosphate buffer pH 7.5 (1:4 w/v) and inoculated to a range of herbaceous test plants.

Symptoms induced by this isolate are summarized in Table 21.

The symptom expression for selected herbaceous plants is described in more detail as follows :

All *Chenopodium spp.* showed only local lesions on inoculated leaves (either necrotic or chlorotic), except for *C. foetidum* which was not infected. *C. amaranticolor* produced small necrotic pin points on inoculated leaves (plate 21) while white necrotic lesions about 2-3 mm. in diameter appeared on inoculated leaves of both *C. album* and *C. murale*. Circular chlorotic spots about 3-4 mm.

in diameter were formed on *C. quinoa* leaves which later developed brown necrotic centres. The symptoms normally appeared on within 5-7 days of inoculation; no systemic infection was detected.

Cucumis sativus : chlorotic spots, about 2 mm., diam., developed on cotyledons within about 7 days, followed by systemic mosaic or vein yellowing on primary leaves (plate 22). Later, the upper leaves became symptomless.

Phaseolus vulgaris "The Prince" showed irregular brown necrotic spots on inoculated leaves only; there was no systemic reaction.

Nicotiana spp. Most species produced chlorotic or necrotic spots about 3 mm. diam. on inoculated leaves (plate 23) except *N. megalosiphon* which developed necrotic spots and vein necrosis. All species produced systemic symptoms, usually a mosaic, while *N. tabacum* developed a line pattern ("White Burley"), or a ringspot symptom ("Xanthi"). Upper leaves, however, were often symptomless (plate 23). *N. megalosiphon* produced a spreading veinal necrosis and distortion of younger leaves.

Plate 21 Small necrotic pin point lesions on inoculated *Chenopodium amaranticolor* leaves infected with CCLV1.



Plate 22 Systemic mosaic and vein yellowing on cucumber leaves infected with CCLV



- Plate 23 a). Chlorotic ringspots on inoculated leaves of *Nicotiana tabacum* "White Burley" with CC1V1.
b). Necrotic ringspots on inoculated leaves of *Nicotiana tabacum* "Xanthi" with CCCLV1.

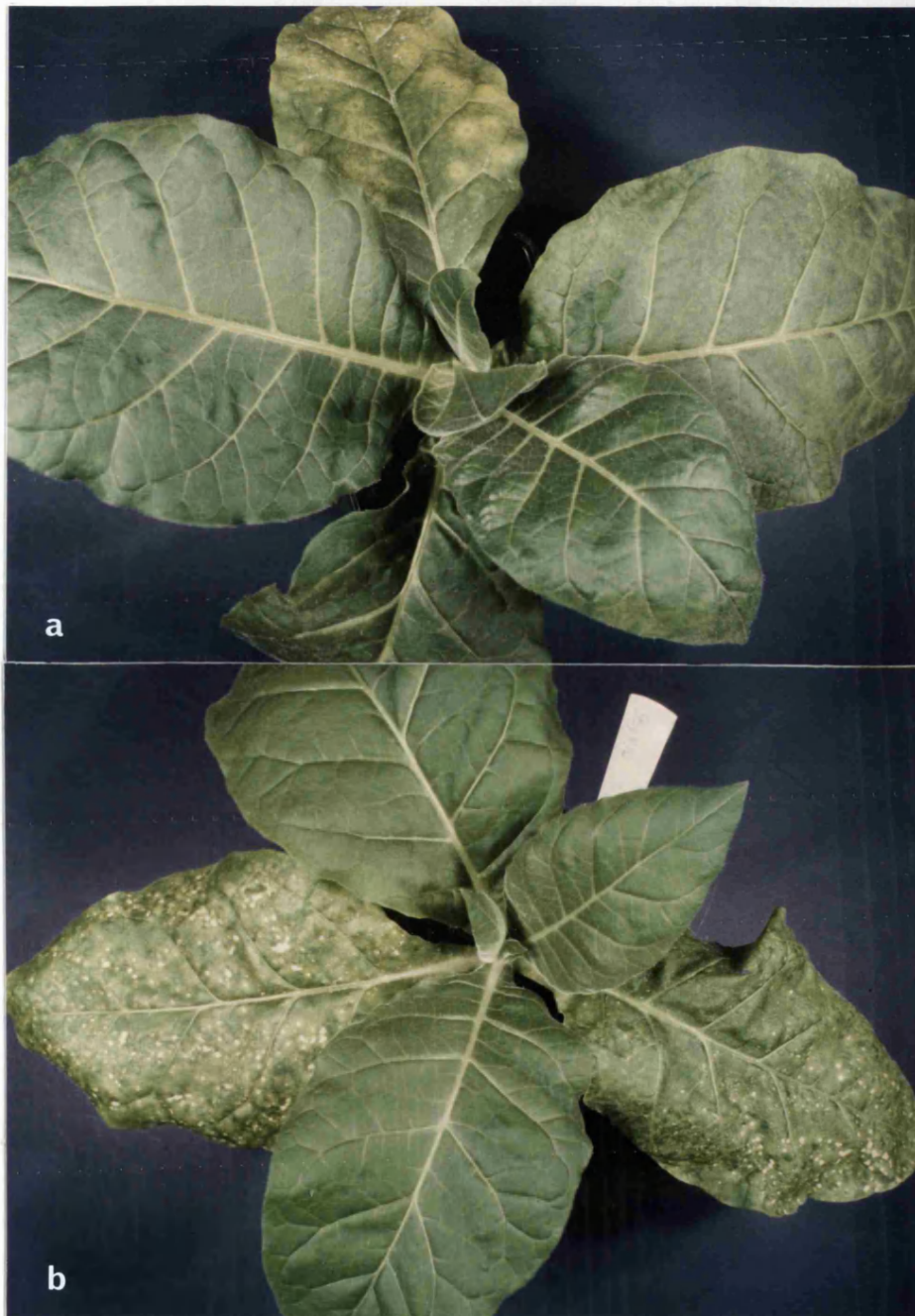


TABLE 21 Symptoms induced on herbaceous hosts by CCLV1 isolate.

<u>Host Plants</u>	<u>Symptom expression</u>
<u>Chenopodium album</u>	NL/O
<u>C. amaranticolor</u>	NL/O
<u>C. foetidum</u>	O/O
<u>C. murale</u>	NL/O
<u>C. quinoa</u>	CL,NL/O
<u>Cucumber sativus</u> "Parisienne Picking"	CL/CM,VY
<u>Datura stramonium</u> var. tatula	C/O
<u>Gomphrena globosa</u>	NL/O
<u>Nicotiana clevelandii</u>	CL/CM
<u>N. debneyi</u>	CL/CM,D
<u>N. glutinosa</u>	CL/CM
<u>N. megalosiphon</u>	NL,VN/VN,D
<u>N. rustica</u>	CL/M
<u>N. sylvestris</u>	CL/CM
<u>N. tabacum</u> " White Burley "	CRS/M,LP
<u>N. tabacum</u> " Xanthi "	NL,RS/M,RS
<u>Phaseolus vulgaris</u> cv. "The Prince"	NL/O

Abbreviations : Local symptom / Systemic symptom

C = chlorotic or chlorosis	L = local lesion
N = necrotic or necrosis	M = mottle or mosaic
RS = ringspot	Y = yellowing
LP = line pattern	VN = vein necrosis
D = leaf distortion	O = no infection
VY = vein yellowing	

4.03 Physical properties of CCLV1 in crude sap.

The sources of inoculum used for studying the in vitro properties of CCLV1 were systemically infected *N. tabacum* "Xanthi" leaves, 10 days after inoculation. Samples were assayed for infectivity on *C. quinoa* in a 6 x 6 Latin square design. The procedures for these tests are described in section 2.09.

4.03 a) Dilution end-point

The DEP for CCLV1 was between 10^{-4} to 10^{-5} .

4.03 b) Thermal inactivation point

The TIP for this isolate was 75°-80°C.

4.03 c) Longevity in vitro

The longevity in vitro for CCLV1 in crude sap at room temperature was between 5 (some infectivity) and 13 days (no infectivity). In contrast sap kept at -20°C. remained infective for one and a half years, the longest period tested.

4.04 Effect of different additives on the infectivity and stability of CCLV1 in *N. tabacum* "Xanthi" extract.

The concentration of each additive for studying virus infectivity and stability was; 70 g/l insoluble polyvinyl pyrrolidone (PVP) (Polyclar AT), 0.01 M ascorbic acid, 0.01 M 2-mercaptoethanol, 20 g/l polyethylene glycol 8000 (PEG), 0.01 M ethylene diamine tetraacetic acid disodium salt (EDTA), 0.01 M sodium diethyl dithiocarbamate (DIECA), 70 g/l soluble PVP and 0.1% (v/v) mercaptoacetate.

0.1 M phosphate buffer pH 7.5 was used as the extraction buffer at a dilution of 1:5 (w/v); phosphate buffer without additives, was used as the comparison (control).

In the test, after 6 hr. at 4°C. aliquots were inoculated to *C. quinoa* in a 9 x 9 Latin square.

Table 22 Effect of different additives on the infectivity and stability of CCLV1 in *N. tabacum* " Xanthi" extracts.

<u>Additives</u> (all in 0.1 M phosphate buffer)	<u>Mean no. lesion per leaf</u> <u>on <i>C. quinoa</i></u>
7% PVP insoluble	43
7% PVP soluble	9
0.01 M ascorbic acid	32
0.01 M 2-mercaptoethanol	17
2% PEG	12
0.01 M EDTA	14
0.01 M DIECA	5
0.1% (v/v) mercaptoacetate	67
control (0.1 M phosphate buffer)	8

The results (Table 22) showed that 7% PVP soluble, 0.01 M DIECA and the control failed to stabilize the virus after 6 hr. period. The chemicals, 0.01 M 2-mercaptoethanol, 2% PEG or 0.01 M EDTA produced lesion numbers which were slightly higher than those produced by the extract made in 0.1 M phosphate buffer, although, results were not significant ($P>0.05$). The best additives in this experiment appeared to be the antioxidant 0.1% mercaptoacetate which was found to be a suitable reducing agent to stabilize the infectivity of cucumber mosaic virus and to prevent the formation of brown components, presumably resulting from oxidation (Tomlinson et al, 1959). Slightly fewer lesions were produced by extracts prepared with 7% PVP (insoluble) and 0.01 M ascorbic acid but these

also stabilized virus infectivity compared to the control. It was not clear why PVP insoluble was more effective than PVP soluble as both presumably from complexes with tannins (Matthews, 1981) It is possible 7% was too high for a soluble formulation.

4.05 Comparision of harvesting time for CCLV1 in tobacco sap.

To obtain high virus concentrations a suitable propagation host must be found as well as the right time to harvest leaf tissue for maximum infectivity. In this experiment, different *Nicotiana* spp. were tested : *N. debneyii*, *N. tabacum* " White Burley ", *N. tabacum* " Xanthi" *N. rustica* *N. glutinosa*

Sap from inoculated leaves were assayed on *C. quinoa* 7, 10 and 15 days after inoculation in a 5 x 5 Latin square design. The procedure was the same as in Section 3.06 b. and the results are shown in Figure 8.

From Figure 8, the infectivity of extracts from inoculated tobacco leaves was generally high 7 and 10 days after inoculation before it declined after 15 days. After 7 days there was little difference between the infectivity of extracts from *N. tabacum* "White Burley", *N. rustica*

and *N. glutinosa* although those from *N. debneyi* were significantly less infective. All species except *N. debneyi* and *N. tabacum* "Xanthi", showed a significant drop in infectivity after 10 days, while all species except *N. rustica* and *N. tabacum* "White Burley ", showed a further significant drop by 15 days.

After taking the infectivity of extracts after 7-10 days and the size of the leaves and subsequent growth of the plants after inoculation into consideration. *N. tabacum* "Xanthi" was chosen for purification studies.

4.06 Serology : Protein A (PA)-Enzyme linked Immunosorbent Assay (ELISA).

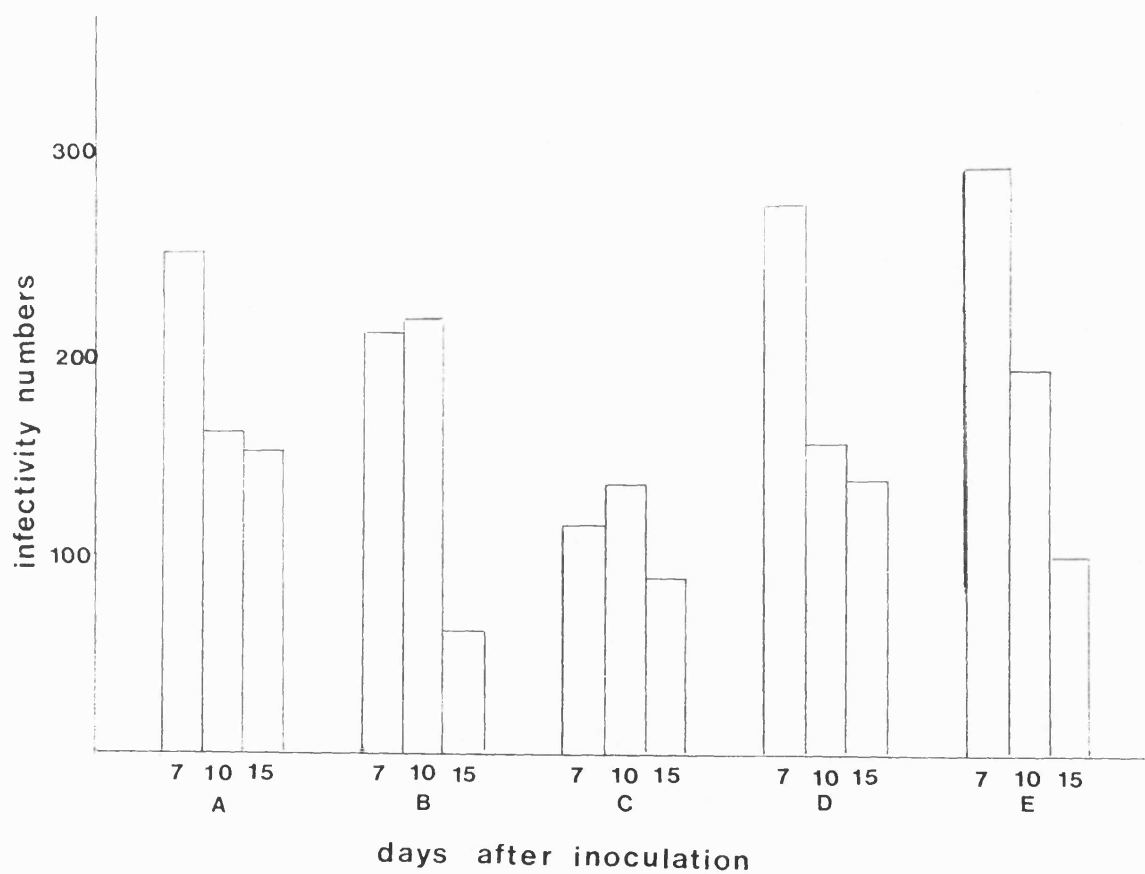
A preliminary assessment of the herbaceous host response of CCLV1 combined with information in the literature (Cooper, 1979) on viruses that infect *Caryopteris spp.* indicated that CCLV1 might be cucumber mosaic virus.

A test was, therefore, set up using a CMV-ELISA system to confirm this diagnosis.

The test was performed according to method of Lommel et al(1982) and was described in Section 2.15 c.

Figure 8 Comparison of harvesting time for CCLV1 in *Nicotiana* sap

- (A) *N. tabacum* "White Burley"
- (B) *N. tabacum* "Xanthi"
- (C) *N. debneyii*
- (D) *N. rustica*
- (E) *N. glutinosa*



An antiserum raised against CMV-*Commelina nudiflora* obtained from American Type Culture Collection was used at a dilution of 1:3000. The enzyme conjugated protein A was used at dilutions of 1:5000, 1:10000 and 1:50000 (v/v). Sap of infected *N. tabacum* "Xanthi" at dilutions of 1:5 (w/v), 1:50 and 1:500 (v/v) was used as the antigen sources. All samples were tested in triplicate in the same manner. The incubation time for substrate was 1 hr.

The results confirmed CCLV1 as CMV and showed that the method was suitable for the detection of CMV in *N. tabacum*. The average absorbance values (410nm) for the ELISA assay are presented in Table 23 .

Table 23 Comparison between different dilutions of enzyme conjugated protein A and dilution of crude sap

<u>Infected tobacco</u>		<u>Mean A 405 nm</u>		
		<u>1:5000</u>	<u>1:10000</u>	<u>1:50000</u>
Diseased :	1:5	0.59	0.29	0.14
	1:50	0.43	0.22	0.13
	1:500	0.19	0.15	0.10
Healthy :	1:5	0.06	0.05	0.04
	1:50	0.04	0.03	0.05
	1:500	0.04	0.04	0.04

The absorbance at each dilution from diseased samples was greater than the mean plus two times the standard deviation of the absorbance of the healthy control a positive-negative threshold often used in ELISA test (Sutula et al., 1986). The 1:5000 dilution of conjugated protein A was chosen for further tests because it provided greatest sensitivity without significantly increasing backgrounds.

A further experiment was done to see if virus could be detected in *Caryopteris clandonensis* using this method. The leaf tissue was diluted at 1:5 (w/v) in antigen buffer and then a 10-fold series was prepared. Infected sap from *N. tabacum* "Xanthi" was used as a positive control.

After 2 hr. incubation, the absorbance was recorded. Absorbance values with *Caryopteris* sap, however, were all in range 0.1-0.2 regardless of whether infected or not. A positive result, however, was obtained only from the wells filled with infected tobacco sap but not *Caryopteris* sap. The failure to detect virus in this experiment was presumably due to the low virus concentration in the leaves when sampled. It was also possible that CMV was unevenly distributed in vivo and some shoots or leaves had not been invaded

by the virus. Davis and Hampton (1986) found the relative CMV concentration on 3-week-old *Phaseolus vulgaris* seedlings was highest in the primary leaf. In addition the failure to detect virus from infected *Caryopteris* sap might be due to the effects of plant inhibitors which interfere with the assay. The absence of an obvious "prozone" effect (Hammond and Lawson, 1988), however, made this unlikely.

4.07 Purification.

The procedures, used to purify various strains of cucumovirus, are nearly as varied as the strain involved. This is particularly true for viruses such as cucumber mosaic virus (CMV) whose biological properties are very variable (Francki and Hatta, 1980). The methods of Tomlinson *et al* (1959), Scott (1963) and Murant (1965), for the purification of CMV, differed from each other, mainly in the type of extraction buffer, and the type of organic solvent for clarification. Tomlinson *et al* (1959) used 8.5% n-butanol while, Scott (1963) chose an equal volume of chloroform, and Murant (1965) applied an equal volume of ether. Later, Tomlinson *et al* (1973) suggested the use of an equal volume of diethyl-ether for CMV strain "Y".

Since CMV particles may be precipitated in some buffers, 0.5 M citrate buffer pH 6.5 containing 0.1% mercaptoacetate was chosen for purification procedure as suggested by Francki(1964). Various clarification solvents were compared to determine their effects on the infectivity, stability and yield of CCLV1.

4.07 a) Comparison of different clarification procedures.

N. tabacum "Xanthi" leaves (200g) harvested 10 days after inoculation with CCLV1 were homogenized in a Wareing blender in 1:2 (w/v) 0.5 M citrate buffer containing 0.1% (v/v) mercaptoacetate pH 6.5. The homogenate was filtered through muslin and divided into four equal portions. An equal volume of chloroform, or diethyl-ether, or n-butanol to 8.5% (v/v), was shaken or stirred (butanol) with one volume of filtered homogenate. As a control, an equivalent volume of buffered extract was left untreated.

The scheme followed is shown in Figure 9. The viral infectivity was assayed by inoculation to *C. quinoa* in a 4 x 4 Latin square. Firstly, after the supernatant from the low g. centrifugation of each treatment had been adjusted to the same volume. Secondly after resuspension

of the high speed pellet in 0.5 ml. of 0.05 M citrate buffer pH 7.

Table 24 Comparison of organic solvents for clarification of CCLV1 in tobacco extracts after low speed centrifugation.

<u>Clarification agent</u>	<u>Appearance</u>	<u>Clarification</u>	<u>Infectivity undiluted</u>
8.5% n-butanol	deep green	+	7
chloroform	pale green	++++	4
diethyl-ether	dirty green	++	13
control	dirty green	++	5

+ = poor clarification, ++++ = good clarification

The results (Table 24) showed that the chloroform-treated extract gave the best clarification. The diethyl-ether and control treatment showed poor clarification while the aqueous phase from n-butanol appeared to be the darkest green. For undiluted samples, diethyl-ether treatment resulted in slightly higher lesion numbers ($P < 0.05$) compared with the other treatment, which were similar to the control.

Figure 9 Comparison of different clarification procedures.
for CCVL1

All stages were performed at 4 °C.

Systemically-infected *N. tabacum* "Xanthi" leaves
Discard material this side

Pulp	<ol style="list-style-type: none"> 1. Leaves homogenised in a Wareing blender with 1:2 (w/v) 0.5 M citrate + 0.1% mercaptoacetate pH 6.5 (low speed blending for 1 1/2 min., then high speed for 1 1/2 min. 2. Squeeze through muslin
Pellet	<ol style="list-style-type: none"> 3. Extract divided into four, then adjusted to 8.5% n-butanol, an equal volume of diethyl-ether or chloroform added to the extract, shake or stir for 20 min. Control was put in crushed ice. 4. Centrifuged at 6000g for 15 min.
Supernatant	<ol style="list-style-type: none"> 5. Each supernatant was adjusted to the same volume with extraction buffer. Samples assayed on <i>C. quinoa</i> 6. Each supernatant spun at 33,000 g for 2 hr.
Pellet	<ol style="list-style-type: none"> 7. Pellets from each treatment resuspended in 0.5 ml of 0.05 M citrate buffer pH 7, overnight 4°C. 8. Spun at 4000g for 25 min at 4°C.
	<ol style="list-style-type: none"> 9. Supernatant = solution of CCLV1 Assayed on <i>C. quinoa</i> to compare virus concentrations in different preparation.

Table 25 Comparison of infectivity after high speed centrifugation

<u>Clarification agent</u>	<u>Infectivity *</u>	<u>Abs 260:280</u>
8.5% n-butanol	250	1.46
chloroform	316	1.70
diethyl-ether	268	1.54
control	176	1.53

* Mean lesion numbers per leaf

After 2 hr. high speed centrifugation at 30,000 g the infectivity of every treatment increased considerably (Table 25). Purification was satisfactory with all of the clarifying agents tested, but only with chloroform was high relative infectivity matched by the best clarification. Moreover, it also gave the highest absorbance ratio of 260:280 at 1.70 similar to that reported by Francki et al (1979) in the CMI/AAB description of CMV.

4.07.b.) Comparison of PEG precipitation and ultracentrifugation for concentration of CCLV1.

The ability of PEG and high speed centrifugation to concentrate CCLV1 from a clarified preparation was studied. The method was compared both with and without chloroform as clarification agent.

Fifty grams of inoculated leaves of *N. tabacum* "Xanthi" harvested after 10 days were extracted and clarified with an equal volume of chloroform as described in section 4.06 a, while the control was left untreated. Both treatments were divided into two equal volumes. To one volume 10% PEG (Mr 8000) and 0.3 M NaCl were added. The mixture was stirred for 1 1/2 hr at 4 °C., then centrifuged at 10,000 g for 20 min. The second volume was centrifuged at 30,000 g for 2 hr. Pellets from each treatment were resuspended in 0.5 ml of 0.05 M citrate buffer pH 7 overnight and clarified by chilled centrifugation for 20 min. at 4000 g.

The UV absorption spectra of the preparations were measured after dilution 1:100 in citrate buffer. Absorption spectrum plots obtained from the spectrophotometer was used to calculate the 260:280 nm. absorbance ratios of the partially-purified preparation (Table 26, Figure 10).

Figure 10 a). Absorption spectra of partially-purified preparations of CCLV1 concentrated using
 (a). PEG precipitation
 (b). high-speed centrifugation
 (both control treatment)

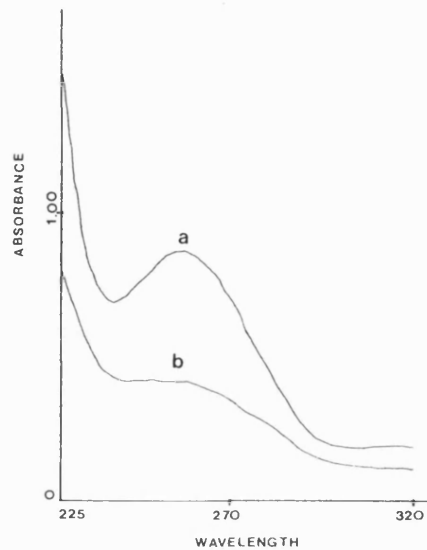


Figure 10 b). Absorption spectra of partially-purified preparations of CCLV1 clarified with chloroform and concentrated using
 (a) PEG precipitation
 (b) high-speed centrifugation

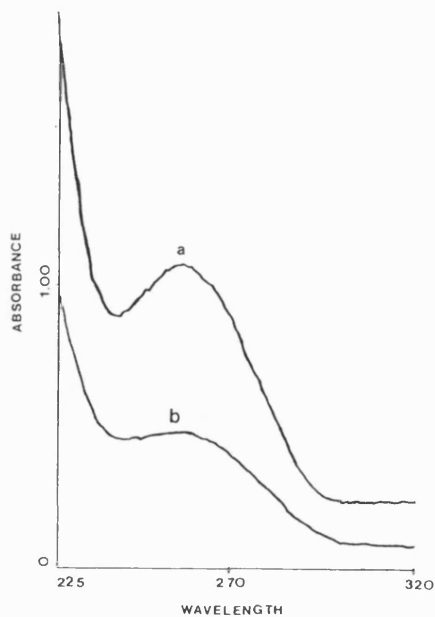


Table 26 Comparison between PEG precipitation and high speed centrifugation for concentration of CCLV1

<u>Concentration method</u>	<u>A 260</u>	<u>A 260:280 ratio</u>
control and PEG precipitation	1.65	1.75
control and high speed centrifugation	0.15	1.53
chloroform and PEG precipitation	1.04	1.73
chloroform and high speed centrifugation	0.46	1.71

The results showed some differences in spectral characteristics between treatments. The control extract with high speed centrifugation only, had the lowest 260:280 ratio and absorbance at 260 nm. suggesting contamination with protein of host plant origin. A higher 260:280 ratio for extracts concentrated by PEG precipitation than by high speed centrifugation indicated that the former was better method for CCLV1 although with clarification the difference was negligible. The ratio of the preparation from chloroform PEG treatment of 1.73 was very close to the ratio of CMV purified preparations given by Francki et al. (1979).

When the A 260 nm values were compared, the use of high speed centrifugation was found to markedly reduce the nucleic acid

(presumably of viral origin) content of the preparations compared to those from the PEG treatment.

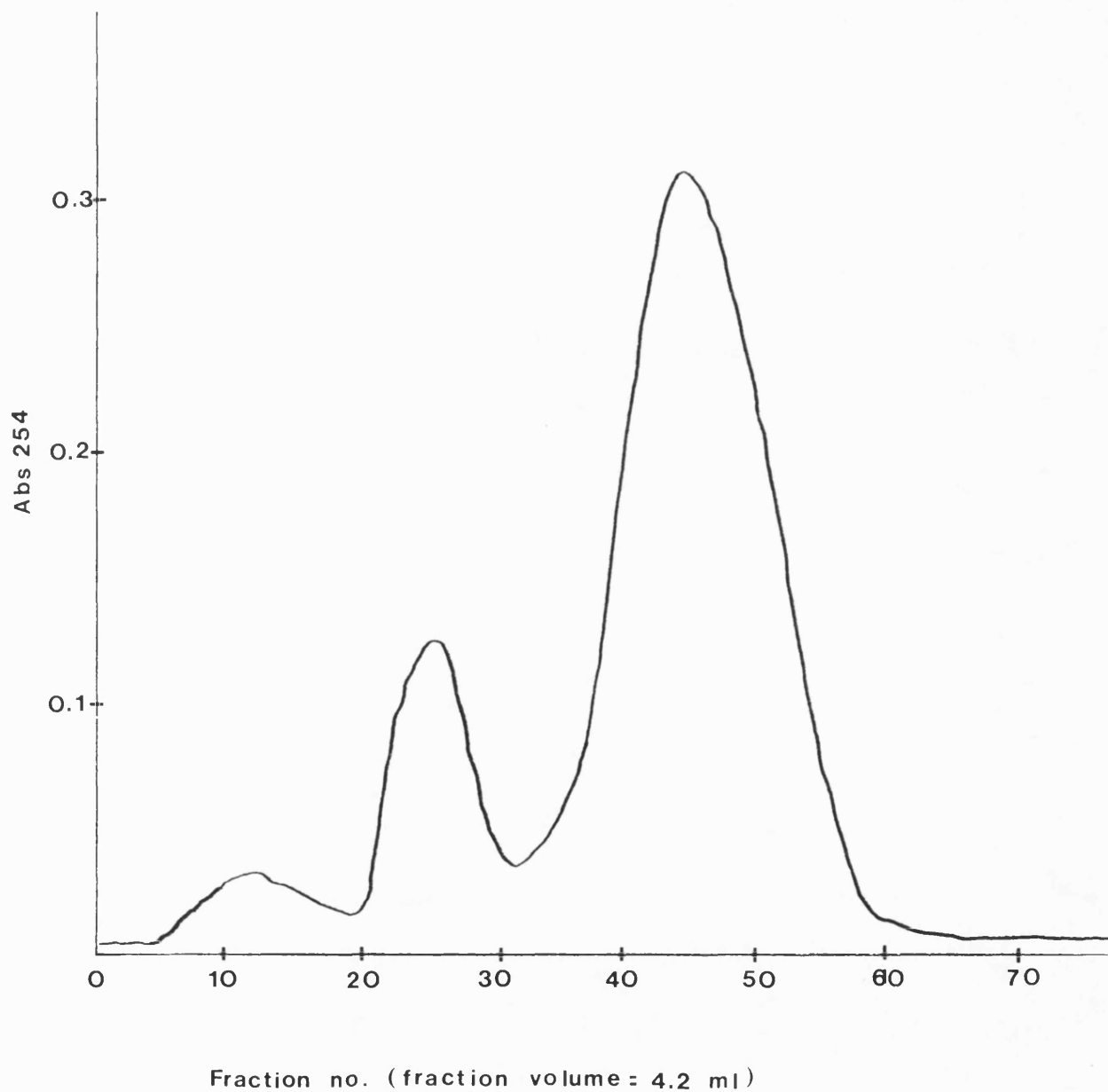
4.07 c) Further purification using permeation chromatography

Attempts were made to further purify preparations of CCLV1 using permeation chromatography.

A hundred and fifty grammes of *N. tabacum* "Xanthi" leaves harvested 10 days after inoculation with CCLV1, were extracted in 0.5 M citrate buffer containing 0.1% mercaptoacetate pH 6.5, clarified with chloroform and concentrated by PEG precipitation.

Samples of 0.5 ml. of partially-purified virus were layered on the CPG (Sigma Chemicals Ltd) column, previously equilibrated with 0.05 M citrate buffer pH 7. The elution profiles showed two major U.V. absorbing peaks (Figure 11). It was possible that the first peak contained a mixture of virus, possibly aggregated, and debris, while the second peak consisted mostly of virus. The pooled fractions of each peak were monitored with a U.V. spectrophotometer (Figure 12) before being concentrated by high speed centrifugation at 30,000 g for 2 1/2 hr. The pellet was then

Figure 11 Elution profile of Abs 254 nm CCLV1 after passage through a CPG column ($V_0 = 78$ ml)



resuspended in 1 ml 0.05 M citrate buffer overnight after a short bench centrifugation and the absorbance determined.

After resuspending the pellet overnight, there was a low absorbance at 260 or 280 nm. indicating a serious loss of virus (Figure 13), possibly because of virus aggregation or the absorption of virus to cell constituents which was reported by Sill et al (1952) for their isolate of CMV. Although, the test was done at least three times, the same result occurred.

4.07 d) Effect of additives on resuspension of CCLV1

Takanami and Tomaru (1969) showed that EDTA prevented CMV-Y from aggregation, and Jones (1976) demonstrated that the use of 0.001 M EDTA in the buffer during purification was essential for a raspberry isolate of CMV . Preparation made by omitting EDTA at any stage contained few particles, were only weakly infective and proved to be very unstable on storage at 4 °C. Takanami and Tomaru (1969) also reported that the tendency of CMV to aggregate appeared to be attributable mainly to small amounts of free divalent cations in plant juice, and that the presence of EDTA completely inhibited aggregation.

Figure 12

Absorption spectrum of undiluted eluant from CPG column of CCV1 partially-purified preparation (a)peak 1(b)peak 2

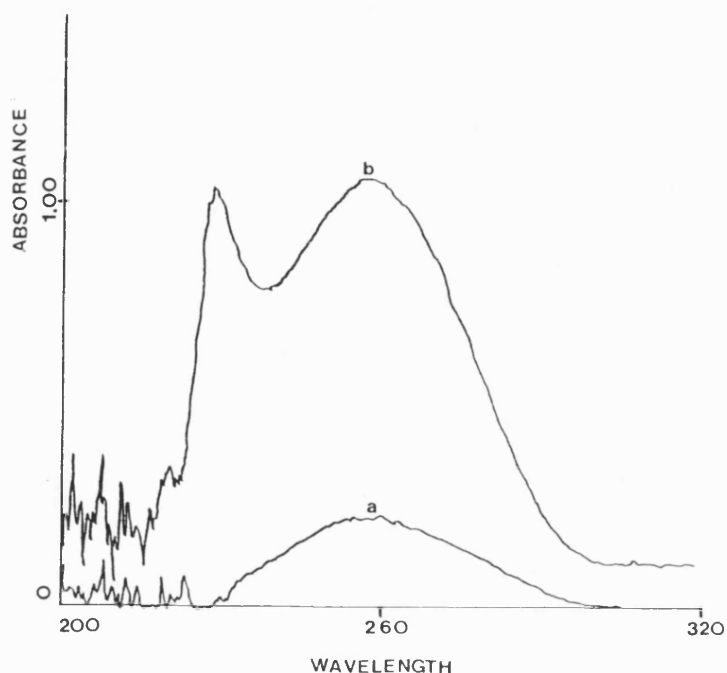
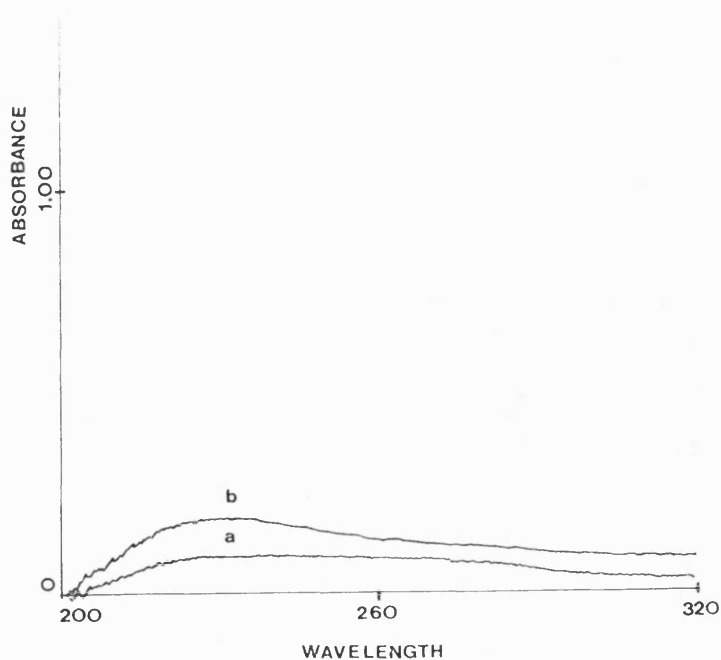


Figure 13

Absorption spectrum of eluant from CPG column after high speed centrifugation diluted in 1 ml 0.05 M citrate buffer (a) peak 1 (b)peak 2



Several purification methods have been used for CMV, most of which are modifications of those devised by Scott (1963). Triton X-100 at 2% (v/v), however, is normally included in the resuspending buffer (Lot et al , 1972. ,Mossop et al, 1976., Walkey, 1985)

A further experiment was performed in which, the virus pellet after PEG precipitation was resuspended overnight in 0.5 ml of either 0.05 M citrate buffer with 0.001 M EDTA or 2% triton X-100 or in 0.05 M citrate buffer only, or in 0.005 M citrate, all at pH 7. The samples were compared for infectivity using 2 sets of *C. quinoa* plants in 4 x 4 Latin square design.

The results are presented in Table 27.

Table 27 Influence of additives in resuspending buffer on infectivity of CCLV1

<u>Treatment</u>	<u>Mean no.lesion per leaf</u>
0.05 M citrate	146
0.05 M citrate and 0.001 M EDTA	56
0.05 M citrate and 2% triton X-100	3
0.005 M citrate	23

The results showed that the presence of 0.001 M EDTA or 2% triton X-100 in resuspending buffer, significantly ($P < 0.05$) reduced the infectivity of CCLV1. The treatment which gave the highest lesion numbers was 0.05 M citrate without any additives. This treatment was significantly better ($P < 0.05$) than the others. On this basis, metal ions did not appear to be responsible for the failure of CCLV1 to resuspend satisfactorily, although it was possible that citrate may also act as a chelator (Matthews, 1981).

The attempt to purify CCLV1 using a CPG column with 0.05 M citrate pH 7.0 as running buffer was repeated using infective preparations that showed a good U.V. spectrum typical for a viral nucleoprotein (260:280 at 1.7). Following CPG chromatography, and high speed centrifugation of column peak fractions the pellets were resuspended overnight at 4°C. After a brief bench centrifugation, the U.V. absorbtion spectrum was taken, and samples assayed undiluted on *C. quinoa*. There was no infection on any of the test plants and the absorbance at 260 nm. was low (< 0.1), the curve was nearly flat with no clear peak at 260 and 280 nm. indicative of nucleoprotein (rf. Figure 13).

The results indicated that most virus was lost between the process of elution from CPG column and resuspension of the pellet after ultracentrifugation. Fixation of particles with 1% formaldehyde or glutaraldehyde before layering on the CPG column made little difference to virus recovery as assessed from the U.V. data. It is possible, that centrifugation of the sample at high speed caused the virus to aggregate or made the particles unstable in some other way. However, this seemed unlikely from the results of a further experiment, in which partially-purified samples were compared for infectivity before and after ultracentrifugation. One half of the sample was centrifuged at 30,000 g for 2 hr., the other half was stored for the same period at 4°C. The centrifuged sample was then resuspended in the original volume of buffer (1 ml) and both samples assayed on *C. quinoa* half-leaves (10 replicates). There was no significant ($P>0.05$) difference in infectivity between the two treatments with lesion mean of 38 (before) and 30 (after) centrifugation. This suggested that virus particles were being adversely affected during column chromatography rather than ultracentrifugation, although the U.V. data from earlier experiments conflicted with this conclusion. Whatever the explanation, purification needs to be studied

further in order to produce an antiserum for this virus isolate.

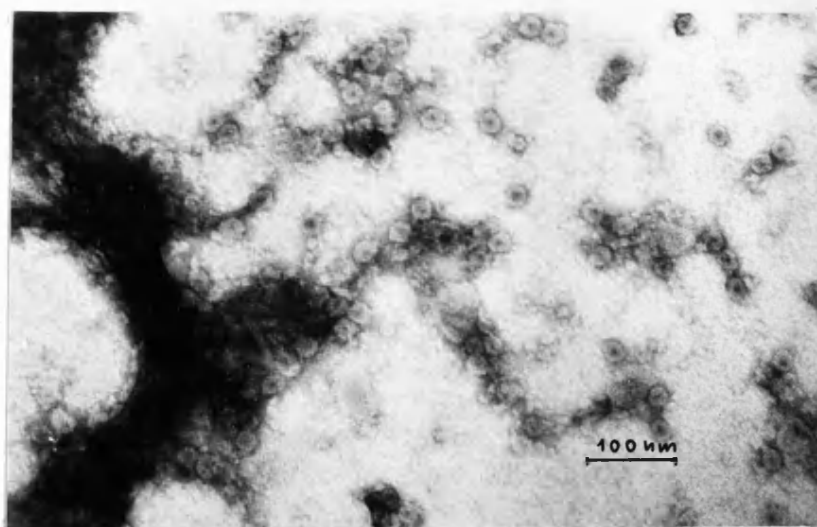
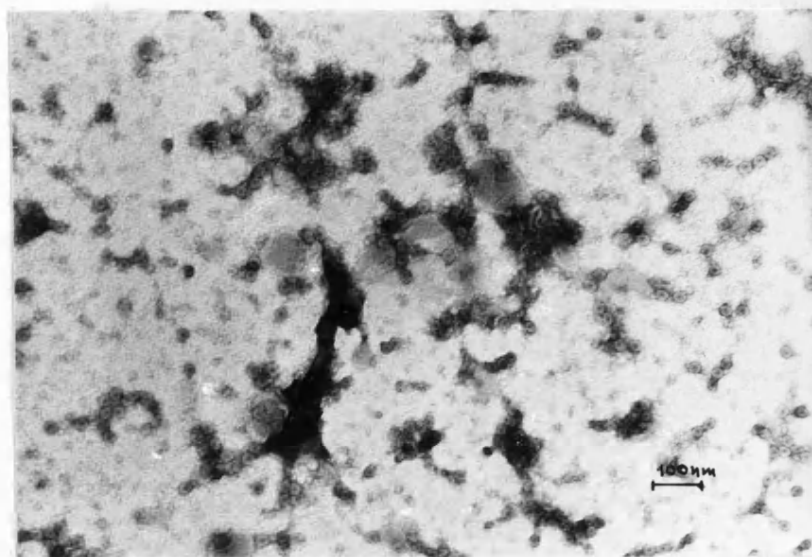
4.08 Electron microscopy

Examination of a partially-purified sample clarified by chloroform and concentrated by PEG precipitation in the electron microscope after staining with 2% uranyl acetate stain pH 5 showed moderate concentrations of intact isometric particles about 28-30 nm. in diameter (plate 24). A prominent central stain-filled area was clearly visible in several particles and is a characteristic of CMV in uranyl stains (Francki, 1972).

4.09 Production of antiserum to host proteins

The separation of a virus from cell components of the host is necessary for the study of some of its properties. Antihost sera (antisera prepared by injecting animals with crude juice of a non-infected host plant) are rarely used as a means of testing for virus purity, yet they can readily demonstrate contamination of apparently purified virus preparations by certain host constituents. The technique was used as early as 1937 by Dunin to prepare antisera against a plant virus which would not react with host juice. Gold (1961) reported that antihost serum which can be used to remove host

Plate 24 Electron micrograph of partially-purified CCLV1 stained with 2% uranyl acetate pH 5. Note the characteristic central stain-filled 'hole' of the particle.



proteins from a virus preparation, improved the purification of tobacco necrosis virus strain.

Using Gold's approach an attempt was made to improve the purification of CCLV1. Healthy *N. tabacum* "Xanthi" leaves were purified as described before (Section 4.06 b) for CCLV1-infected tobacco leaves, using chloroform clarification and PEG precipitation of host protein. The partially-purified healthy leaf sample was dialysed for 24 hr. against 3 litres of 0.05 M citrate buffer containing 0.85% (w/v) sodium chloride. The buffer was changed after 12 hr.

Antiserum to host proteins was prepared by injecting a male brown rabbit (Sandy Lop) with 0.5 to 1.0 ml. of purified sample (0.1-1 mg/ml) adjusted to 0.15 M with sodium chloride. The rabbit was first bled to obtain a normal pre-immune serum sample and then given one intravenous injection, followed at 7-day intervals by 6 intramuscular injections of a mixture of 0.5 ml sample and 0.5 ml of Freund's complete adjuvant. The rabbit was first bled 1 week after the third injection and then bled every week to test the serum titre using gel double-diffusion test. After the first bleed the homologous titre of the serum was 1:16 but by the fourth bleeding, the titre had

declined from 1:64 to 1:32. An intravenous booster injection was therefore, given and the titre rose to 1:128. At this point the last bleed was done aseptically by cardiac puncture.

4.10 Further purification of CCLV1 using antihost serum

The antiserum was used at a dilution of 1:8 in the test. One volume of partially-purified virus sample was mixed with 5 volumes of host-antiserum in glass tube of 3 mm. diameter and left undisturbed at 4°C. for 3 hr. A turbid band formed in solution which was clarified by centrifugation for 5 min. at 5000 g. The clear supernatant was pipetted off and kept at 4 °C. overnight to allow further precipitation to occur. The solution was then clarified again before assay. The antihost serum-treated sample was compared with a similar sample of partially-purified virus, diluted with the same volume of 0.05 M citrate buffer in a half leaf comparison on *C. quinoa*. No lesions were produced for the sample treated with host-antiserum while the control gave a mean of 79 lesions per half-leaf (6 replicates). The complete loss of infectivity could be due to the presence of a substance in normal serum which caused irreversible denaturation of RNA. Matthews (1981) reported that nucleases in

rabbit serum may result in inactivation of viruses not fully protected against enzyme attack. Jacrot et al. (1977) reported that in CMV the packing of the protein subunits was such that about 15% of the surface could be made of holes, which would expose the RNA to inactivating agents.

A further experiment was done after the gamma-globulin had been purified using Na_2SO_4 precipitation. For this a partially-purified virus preparation was divided into 4 parts and mixed (1:5) with normal pre immune serum, antihost serum, purified antihost serum or buffer.

The results are shown in Table 28.

Table 28 Infectivity of partially-purified virus treated with host-antiserum

<u>Treatment</u>	<u>Mean no. lesion per leaf</u> (8 replicates)
control purified virus and buffer	95
control purified virus and normal serum	0
control purified virus and serum	0
control purified virus and serum after salt precipitation	63

Purification of gamma-globulin from crude host-antiserum significantly reduced the loss of virus infectivity. This may have been due to

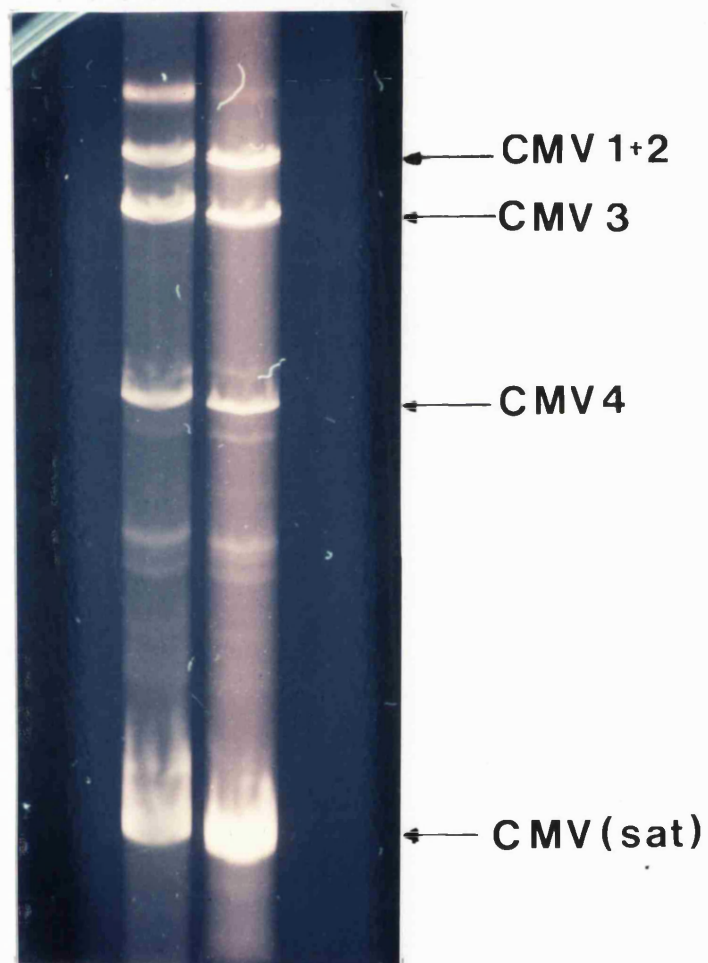
removal of the inhibitory properties of crude serum. Further studies may improve the potential value of this technique for purification of CCLV1.

4.11 Double-stranded RNA analysis

Double-stranded RNAs (dsRNA) was extracted from *Nicotiana debneyi* and *N. tabacum* "Xanthi" infected with CCLV1 (as section 2.14) and analyzed by 6 % polyacrylamide gel electrophoresis for 4 hr. at 5 mA/tube. As a control dsRNA was extracted from "Xanthi" infected with a lettuce isolate of CMV (CMV-W) and analysed in parallel with CCLV1.

Comparison of dsRNA patterns from CCLV1 or CMV-W infected tobacco showed very similar segment profiles and supported the serological identification of CCLV1 as CMV. Four major dsRNA bands were present with both isolates, although dsRNA of RNA 1 and RNA 2 had not clearly separated. The dsRNA of RNA 3 and RNA 4 were clearly visible as well as a "minor" possibly subgenomic dsRNA band after RNA 4 (plate 25). Both isolates had a low Mr segment which was in highest concentration relative to other dsRNA segments, a characteristic of CMV satellite dsRNA (Waterworth and Kaper, 1981). All except the top band survived subsequent incubation in DNAase and in high salt RNAase, but

Plate 25 Ethidium bromide stained polyacrylamide gels showing dsRNA extracted from *N. tabacum* "Xanthi" infected with CCLV1 (left) and CMV "W" (lettuce isolate) (right)



digested in low salt RNAase, confirming their dsRNA nature.

4.12 Return inoculation of CCLV1 to *Caryopteris clandonensis* seedlings

Five apparently virus-free *Caryopteris clandonensis* seedlings, were kept in the dark for 24 hr and inoculated with an infective CCLV1 partially-purified preparation. Inoculated leaves, uninoculated leaves and shoot tips from each seedling were indexed on *Chenopodium quinoa* after 1 month and 4 months, but no virus was detected. Further monitoring of inoculated plants for infection should be done to allow time for low levels of virus to multiply.

4.13 Discussion

The present study indicated on the basis of serological tests, dsRNA similarity, host range and host reaction, CCLV1 was a strain of CMV. This is apparently the first record of CMV in *Caryopteris* in the U.K. CMV was reported to infect *Caryopteris clandonensis* in GDR by Schimanski et al (1977). However, their isolate was not characterised and could not be compared with CCLV1.

The properties of the virus did not

differ significantly from those reported for cucumovirus group although the TIP of 75°-80° C. was slightly higher than some published values. Hollings and Stone (1971) reported a TIP of about 70°C. for CMV and 50°-60°C. for peanut stunt virus and tomato aspermy virus (member of the same group). Francki and Hatta (1980) reviewed data on CMV taken from 20 papers published between 1927-1979 and reported values for TIP from less than 55° to 75°C. Nevertheless, the DEP for CCLV1 (10^{-4} - 10^{-5}) was the same as that reported by Gibbs and Harrison (1970) and Mink (1972) for cucumber mosaic virus.

Some strains of CMV are known to be difficult to purify (Hollings and Stone, 1981), because of low yields of virus in test plants or, alternatively, instability during purification. Although Tomlinson et al. (1973) found EDTA useful in purification of CMV, in the present study the incorporation of 0.001 M EDTA did not prevent degradation of virus, although the concentration of the chelating agent may have been important. Morris-Krsinich et al., (1978) found that a purified preparation of a CMV isolate from *Daphne odora* "Leucanthe" precipitated on storage overnight at 4°C in 0.005 M sodium borate buffer, pH 9.0. Incorporation of 0.001 or 0.005 M EDTA reduced aggregation of virus, while using of 0.01 or 0.02 M

EDTA did not prevent precipitation. It may be useful to investigate a wider range of concentration as well as different chelating agents for CCLV1 but it may be that isolates of CMV from woody ornamentals are more difficult to purify (eg.Perkins, 1987).

Permeation chromatography on columns of controlled pore glass was chosen because it is comparatively gentle treatment for viruses. Barton (1977) used it successfully to purify tomato aspermy cucumovirus. However, the infectivity of the CMV isolate from infected *Caryopteris* plants did not appear to survive passage through the column. If CCLV1 is to be characterised further, more work will be needed to identify a suitable method for virus purification. In preliminary experiments precipitation of host contaminants from virus preparations with antihost serum (gamma-globulin) appeared to have potential.

Tomlinson (1975) proposed an epiphytological model the spread of for CMV that involved the interaction of weeds, insect movements, crop-planting time and related factors. Quiot et al. (1976) identified CMV in 9 perennial and 15 annual diseased weed species that were growing adjacent to fields of affected tomatoes. Quiot et al (1979) isolated CMV from 39 weed species and learned that

certain strains were regularly obtained from specific species, whereas, other strains were associated with other weed species. The most notable characteristic of the natural spread of CMV is that it is transmitted by numerous species of aphids. Fritzsche et al (1972) listed some 75 species that transmitted CMV, especially *Aphis gossypii* and *Myzus persicae*. In the present study, infection of *C. clandonensis* may have arisen either through clonal propagation of infected material, or through vector movements in the field either at Long Ashton or at the site of the contributing nursery. Aphid vectors appear to be able to survive on *Caryopteris* spp. for much of the year, inducing inflorescence galls during summer (Darlington, 1975), so the potential for field spread is always present. The apparent negligible effects of CMV on growth of *Caryopteris*, however, would make specific control measures of doubtful value.

PART B : VIRUS INFECTED *CARYOPTERIS CLANDONENSIS*
CLONE 13

A virus designated CCLV2 was isolated from leaves of *Caryopteris clandonensis* clone 13 plant grown at Long Ashton Research Station as part of the clonal selection scheme assessment programme (Section A). The single infected plant was very stunted in growth compared with non-infected plants of the same clone (plate 26), and was less than about half the height of normal plants. Growth was uniformly reduced over the plant, internodes were shorter and leaves reduced in size. The plants flowered very late compared with normal plants, although fertile seeds were produced.

4.14 Host range study and identification

For host range studies the inoculum source was a well separated single local lesion from *C. quinoa*, maintained in systemically-infected *C. quinoa*. Symptoms on test plants are given in Table 29 .

The reactions of selected hosts are described further below:

Plate 26 Stunted *Caryopteris clandonensis* clone 13
infected with CCLV2 in the field at Long
Ashton Research Station.



Table 29 Symptoms induced on herbaceous hosts by
CCLV2 isolate

Host Plant	Symptom Expression
<i>Apium graveolens</i> cv. "Dulce"	SI/M
<i>Beta vulgaris</i>	SI/SI
<i>Chenopodium album</i>	CL/M, VY, D
<i>C. amaranticolor</i>	CL/M, VY, D
<i>C. murale</i>	CL/D, TN
<i>C. quinoa</i>	CL-RS/M, VY, D
<i>Cucumis sativus</i> cv. "Parisian Pickling"	CL/M
<i>Coriandrum sativum</i>	SI/SI
<i>Daucus carota</i> cv. "Chantenay Red"	SI/SI
<i>Nicotiana clevelandii</i>	SI/M
<i>N. glutinosa</i>	CL/SI
<i>N. debneyi</i>	SI/SI
<i>N. megalosiphon</i>	SI/SI
<i>N. rustica</i>	SI/SI
<i>N. sylvestris</i>	SI/SI
<i>N. tabacum</i> "White Burley"	SI/SI
<i>N. tabacum</i> "Xanthi"	SI/SI
<i>Phaseolus vulgaris</i> cv. "The Prince"	O

Abbreviations : local reactions/systemic symptom

C = chlorotic or chlorosis	M = mottle
L = local lesion	F = flecking
VN = vein-necrosis	D = distortion
O = no infection	RS = ring spot
VY = vein-yellowing	TN = tip necrosis
SI = symptomless infection	

Chenopodium amaranticolor. The isolate induced indistinct chlorotic local lesions on inoculated leaves within 7 days, followed by systemic mottling, 2 weeks after inoculation. Young leaves were dwarfed and misshapen and plants stunted.

C. murale. Chlorotic lesions appeared on inoculated leaves after 7-10 days. The lesions later become necrotic. The plant tip also showed necrosis and leaves were distorted (plate 27).

C. quinoa. Within 7-10 days inoculated leaves showed diffuse chlorotic local lesions (plate 28 a), while uninoculated leaves produced a systemic yellow mottle within 2 weeks (plate 28 b). Young leaves became distorted and the tip necrotic.

Cucumis sativus. Diffuse chlorotic local lesions developed on cotyledons after 10 days, whereas true leaves showed systemic interveinal chlorosis and mosaic (plate 29). This systemic symptom, however, appeared only in winter. Leaves were symptomless in summer but still contained virus.

Most of the *Nicotiana spp.* were symptomlessly infected.

Plate 27 Necrotic lesions on inoculated leaves and shoot tip necrosis and leaf distortion on *C. murale* infected with CCLV2.



Plate 28 a) Diffuse chlorotic lesions on inoculated leaves of *C. quinoa* infected with CCLV2



Plate 28 b). Leaf mottling on uninoculated leaves of *C. quinoa* infected with CCLV2.



Plate 29 Systemic interveinal chlorosis and mosaic on true leaves of cucumber infected with CCLV2.



4.15 Seed transmission of CCLV 2

Seeds were collected from the dry flower heads of *C. quinoa* plants systemically-infected with CCLV2 isolate. Two hundred seeds were taken at random from bulked samples of infected or healthy seeds. The seeds were sown as described in Chapter 2.02 b. and the proportion which germinated was determined.

Germination under mist took c. 3 days for both infected and healthy seeds, collected at the same time. The germination percentage was 85 and 81 for healthy and diseased seeds, respectively. To look for symptoms in seedlings, they were pricked out at a density of 4 plants per pot. After 4 weeks about 85% of seedlings from plants infected with CCLV2 showed chlorotic mottle and leaves appeared malformed and distorted (plate 30). Seedlings with symptoms were assayed by backtesting onto two *C. quinoa* seedlings. All backtests were positive.

After 4 weeks growth, leaf areas and the height of progeny plants were measured. The mean leaf area was 1.89 cm.² for leaves on seedlings from infected plants and 12.02 cm.² for leaves of healthy seedlings grown under the same conditions (50 replicates; $P < 0.001$). The mean

heights were 3.46 cm. and 12.88 cm. for seedlings from infected and healthy seedlings (60 replicates; $P < 0.001$), respectively.

4.16 Physical properties of CCLV2 in crude sap

For determination of the properties in vitro crude sap prepared by grinding infected *C. quinoa* leaves in 0.01 M phosphate buffer pH7.5 (1g leaf to 2 ml buffer) was used. The infectivity of the treated sap, was assayed on *C. quinoa* seedlings (5 replicates).

4.16 a) Dilution end point

The DEP of CCLV2 was between 10^{-3} to 10^{-4} .

4.16 b) Thermal inactivation point

The CCLV2 isolate had a TIP of 50° - 60°C .

4.16 c) Longevity in vitro

Crude sap stored at room temperature (20° - 25°C .) remained highly infectious after 40 days. Frozen sap kept at -20°C . was still infective after one year, the longest period tested.

Plate 30 *C. quinoa* seedling progeny from plants infected with CCLV2. Seedlings show chlorotic mottle and leaf malformation.



4.17 Serological identification of CCLV2

The data collected on the host range reaction of CCLV2 and its high levels of seed transmission and the isolated patchy distribution of disease in the field suggested that CCLV2 might be a nepovirus. To confirm this, CCLV2 was tested in agar gel double diffusion against antisera to strawberry latent ringspot virus (SLRV), arabis mosaic virus (ArMV), and raspberry ringspot virus (RRV). These viruses are three of the commonest nepoviruses found in the U.K. (Murant, 1981).

Undiluted sap from systemically-infected *C. quinoa* leaves was used as antigen source and sap from healthy *C. quinoa* was used as a control. Antigen was placed in the outer wells, and antisera used undiluted or diluted 1:32 in phosphate buffered saline was placed in the central well.

After overnight incubation of the gel frame, at room temperature, a single precipitation line developed between CCLV2 and antiserum to SLRV at 1:32. No precipitation lines developed with healthy control samples at this dilution, or with ArMV and RRV. This test confirmed CCLV2 as an isolate of SLRV.

4.18 Soil bait test

Seedlings of *Chenopodium quinoa* were transplanted into FP9 square pots (plantpax) containing soil samples taken from the rhizosphere of the stunted *Caryopteris clandonensis* plant (clone 13) at Long Ashton Research Station. Within 4 weeks, the young leaves of the bait plants showed systemic mottle and leaf distortion. The infected bait plants flowered prematurely and died after about 10 weeks. Back inoculation to *C. quinoa* and agar gel double diffusion test confirmed the presence of SLRV.

This indicated that there was a population of viruliferous vector nematodes in the soil around the infected shrubs. Lister (1964) and Taylor and Thomas (1968) reported that two nematodes of the genus *Xiphinema* transmitted SLRV; *X. diversicaudatum* and *X. coxi*. Both adults and juveniles are capable of spreading the disease and it seems that in *X. diversicaudatum* virus particles are reversibly associated with the cuticular lining of the lumina of the odontophore and the oesophagus (Murant, 1974). Previously the land used to grow woody ornamentals at Long Ashton had been an orchard which had probably allowed large vector populations to build up on that site.

Some of these nematodes may have acquired SLRV from a previous crop or weed and transmitted it to healthy *Caryopteris*. It is also possible, however, that vector nematodes simply acquired the SLRV from *Caryopteris* which had been infected prior to planting at Long Ashton.

4.19 Purification of CCLV2 isolate

Prior to purification, the virus was bulked up in *C. quinoa* seedlings. This host was chosen because the virus went systemic and it produced clear symptoms of infection. Although, other workers have used cucumber as a purification host for SLRV (Lister, 1964; Allen et al., 1970; Mayo et al., 1974; Hicks, 1979) or *N. clevelandii* (Mayo et al., 1982), *C. quinoa* was used by Perkins (1987) for an isolate from horse chestnut. Inoculated and systemic leaves were harvested two weeks after inoculation. Virus was purified by Fulton's (1959) method using hydrated calcium phosphate (HCP) and was compared with n-butanol method of Tomlinson et al. (1959). After clarification, virus was precipitated by polyethylene glycol (PEG) Mr 6000. The extraction and resuspending buffers were the same as those used by Mayo et al., (1982) for purification of SLRV from strawberry.

Leaf samples of 100 g. were homogenised in a Wareing blender with 150 ml of cold 0.07 M sodium phosphate buffer containing 0.1% 2-mercaptoethanol and 0.01 M EDTA pH 7, and filtered through muslin. One portion of the filtrate was blended with HCP (0.6 g/1g leaves) for 5 min., to mix it together, and an equal volume was adjusted to 8.5% n-butanol and stirred for 45 min. Both samples, were clarified to pellet denatured host material by low-speed centrifugation (10 min. at 10,000 g). The supernatants were collected separately and adjusted to the same volume before 10% PEG and 0.17 M NaCl was added. These were stirred into the solution at 4°C. for 2 1/2 hr on a magnetic stirrer. The virus was pelleted by centrifugation at 12,000 g for 20 min. then resuspended overnight at 4 °C. in a total of 2.5 ml of 0.017 M sodium phosphate buffer. Resuspended pellets were clarified by centrifugation for 15 min. at 4000 g., followed by high-speed centrifugation at 40,000 g. for 2 1/2 hr. Each high speed pellet was left overnight in 1 ml. buffer before clarification at 3000g for 10 min.

Resuspended high speed pellets were diluted and assayed on 5 *C. quinoa* seedlings per treatment. The results are presented in Table 30.

Table 30 Infectivity of partially - purified preparations of CCLV2

<u>Treatment</u>	<u>Dilution series of inoculum</u>						
	1:8	1:16	1:32	1:64	1:128	1:256	1:512
n-butanol	+	+	+	+	+	+	-
HCP	+	+	-	-	-	-	-

+ = plants infected : - = no infection

The results indicated that preparation clarified by n-butanol were significantly more infectious than those clarified by HCP. Furthermore, the degree of clarification was superior with n-butanol. In further studies 8.5% n-butanol was used for clarification. Mayo et al., (1982) also successfully used this solvent to clarify extracts of *N. clevelandii* infected by strawberry isolate of SLRV. Most partially-purified preparations clarified by n-butanol had precipitation end-point between 10^{-2} to 10^{-3} in gel diffusion tests.

Limited attempts were made to purify SLRV further using CPG chromatography. U.V. absorption data of the main peak from the column showed a spectrum typical of viral nucleoprotein. After high speed centrifugation, however, the resuspended pellets were not infective on *C. quinoa*

seedlings and very few virus-like particles could be observed in the electron microscope.

The reason for the apparent loss of virus was not determined. Some samples of distilled water used to prepare resuspension buffer, however, were subsequently found to be contaminated with bacteria. As a precaution all future reagents and buffers were prepared in sterilized distilled water.

4.20 Serology

An antiserum to CCLV2 was produced using a butanol clarified partially-purified virus preparation. Initially one intravenous injection with 1 ml. of purified sample adjusted to 0.15 M with respect to sodium chloride was given. Then, an intramuscular injection of a 1:1 mixture of partially-purified virus and Freund's incomplete adjuvant was given every week until an adequate titre was reached (as determined by gel-diffusion). Each injection contained from 0.1 to 1 mg/ml of virus.

Antiserum to CCLV2 had a maximum homologous titre of 1:128 9 weeks after the first bleeding. With this antiserum an healthy plant reaction was apparent at 1:4 or below but not at higher dilutions. This indicated that purification

failed to remove all contaminating host proteins, prior to antiserum production.

4.20 a) Detection of CCLV2 in *Caryopteris* sap using ELISA test

The double antibody sandwich method was used for this experiment was based on that described by Clark and Adams (1977). The method for preparing partially-purified gamma - globulin and the conjugate from SLRV antiserum was the same as that described in Section 2.15 c. Tests were conducted in a polystyrene microtiter plates. The precoating globulin and conjugated globulin were used at 1 µg/ml. and 1:500 dilution of the stock solution, respectively. Leaf samples were homogenised in antigen buffer and after filtration through two layers of muslin a dilution series was made in the same buffer. After addition of the substrate, the absorbance of p-nitrophenol was measured at 410 nm after 1 hr. incubation at room temperature. A substrate buffer control was used to "blank" the plate. The effect of dilution of *Caryopteris* sap on absorbance at 410 is presented in Table 31 .

Table 31 ELISA absorbance values (A 410 nm) of dilution of *Caryopteris* sap (mean of three replicates)

Treatment	A 410 nm					
	Dilution of infected sap					
	1:5	1:10	1:50	1:100	1:500	1:1000
Infected	0.43	0.60	0.61	0.51	0.27	0.23
Healthy	0.09	0.08	0.12	0.08	0.07	0.06

Based on absorbance thresholds of at least twice the mean of healthy controls (Sutula et al., 1986) the results showed that there was a satisfactory discrimination between infected "positives" (P) and healthy "negatives" (N) at all sap dilutions. It was noticed, however, that the P:N ratio increased from about 5 at 1:5 to 7-8 at 1:10 shown as an increase in absorbance. This increase suggested the presence of an inhibitor in *Caryopteris* sap which interfered with the absorption of virus to the coated plate at low sap dilutions. This "prozone" effect has been observed for some woody hosts in ELISA tests before (Clark and Adams, 1977; Perkins, 1987).

4.21 Estimation of capsid protein relative molecular mass of CCLV2

Protein was prepared by heating partially-purified preparation (0.5 mg/ml) of CCLV2 in 0.01 M sodium phosphate, pH 7, containing 1% SDS and 1% 2-mercaptoethanol in a waterbath at 100°C. for 5 min. Electrophoresis was in 7.5% polyacrylamide gels containing 0.1% SDS (Maizel, 1968). Protein bands were detected by staining with 0.25% Coomassie blue in methanol (50% v/v) and 7% (v/v) acetic acid : gels were destained in distilled water. Protein preparations examined by electrophoresis produced 2 prominent bands of Mr 42,000 and 27,500 (plate 31). The figures reported for relative molecular mass of SLRV capsid protein is 44,000 and 29,000 (Murant, 1981) which are within the \pm 10% accuracy reported by Weber and Osborn (1969) for SDS polyacrylamide electrophoresis.

4.22 Electron microscopy

Leaf squash preparations of infected *N. clevelandii* stained in 2% PTA pH 6.5 contained tubules with isometric particles (plate 32).

Isometric particles about 26 nm. in diameter were also observed in partially-purified preparations in the same stain.

Plate 31 Coat-protein analysis of CCLV2 by SDS polyacrylamide gel electrophoresis with marker protein, from left to right CCLV2, serum albumin (68,000), GPD (36000) and β -lactoglobulin (18400).

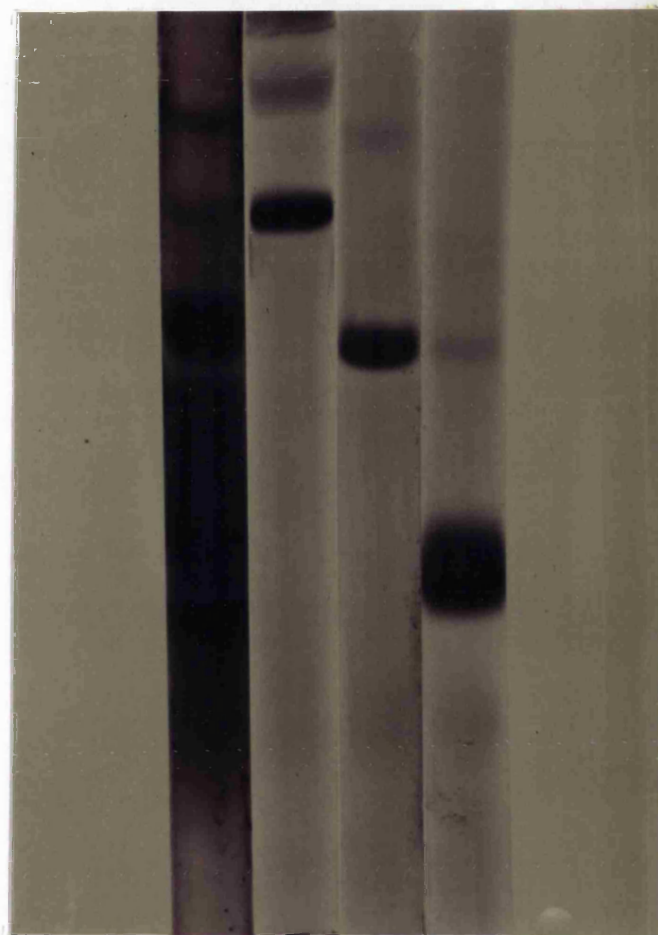
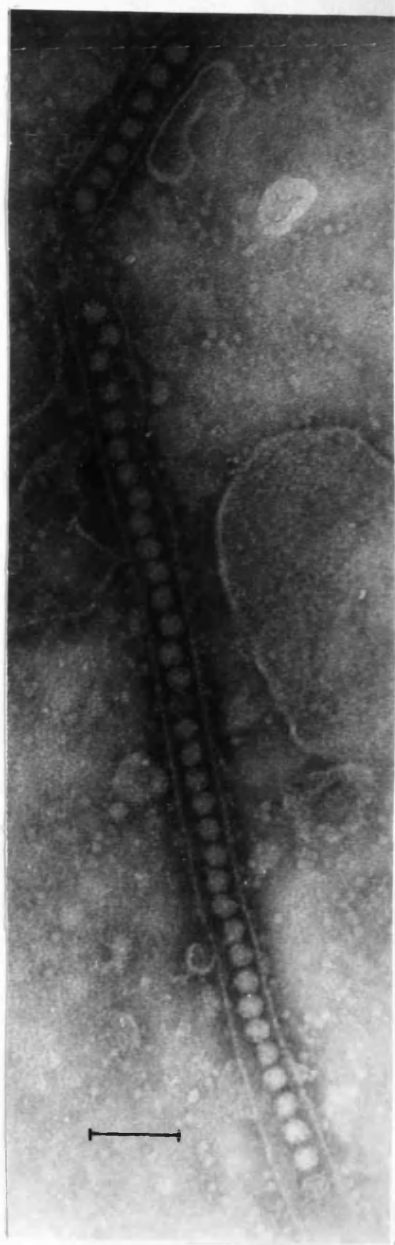


Plate 32

A tubule with isometric particles from leaf squash preparations of CCLV2-infected *N. clevelandii* (stained in 2% PTA pH 6.5). Bar represents 100 nm.

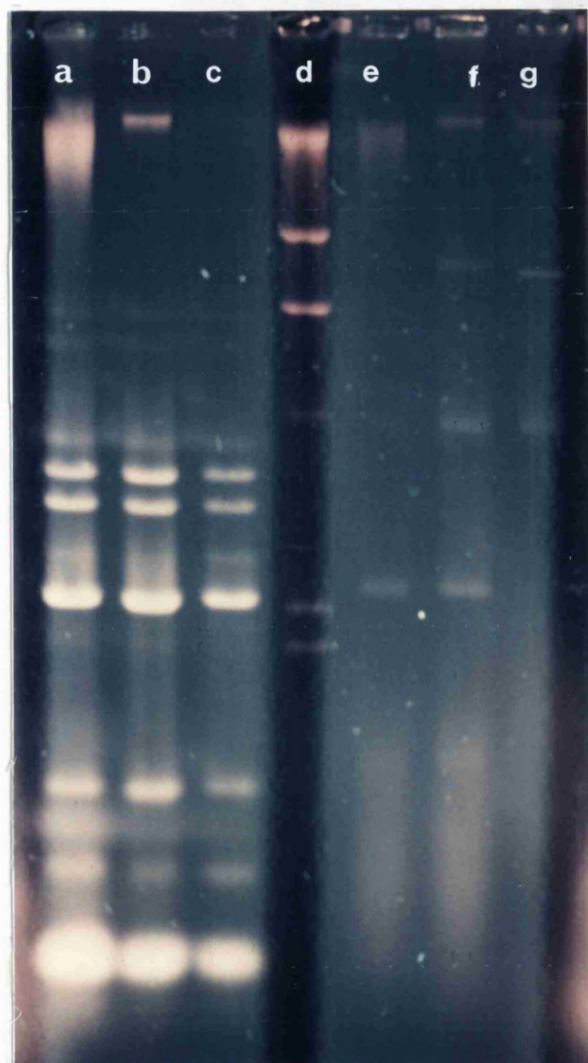


4.23 Double-stranded RNA analysis.

Systemically-infected leaves from mature *C. quinoa* were used for the isolation and analysis of dsRNA (Section 2.14). In a separate extraction dsRNA was also isolated from seedlings of *C. quinoa* infected through the seed. The samples were prepared for electrophoresis by resuspending centrifuged ethanol precipitates in electrophoresis buffer and aliquots of glycerol.

Three major dsRNA segments were detected in extracts from *C. quinoa* infected with CCLV2 with approximate Mr values for the dsRNA of 5.2×10^6 (RNA-1), 2.6×10^6 (RNA-2) and 1.4×10^6 (RNA-3)(plate 33), giving genomic ssRNA values of 2.6, 1.3 and 0.7×10^6 respectively. These values are similar to those reported by Murant (1981) for SLRV, the two heavier segments representing the genomic RNA 1 and 2. The RNA 3 may represent a smaller satellite RNA, found for several definite nepoviruses as well as for SLRV (Mayo et al., 1974). It was noted that in samples from seedlings infected via seed the low Mr RNA 3 had disappeared.

Plate 33 Ethidium bromide stained 0.6% agarose gel showing dsRNA extracted from :-



- (a) *Nicotiana tabacum* "Xanthi" infected with CMV (SV1 isolate)
- (b) *Nicotiana tabacum* "Xanthi" infected with CMV (SV2 isolate)
- (c) *Nicotiana tabacum* "Xanthi" infected with CMV 'W' (lettuce isolate)
- (d) lambda DNA digested with Hind III
- (e) SLRV (parsnip isolate) from *C. quinoa*
- (f) SLRV (*Caryopteris* isolate) from *C. quinoa*
- (g) SLRV (*Caryopteris* isolate) from *C. quinoa* seedlings infected through seed.

4.24 Return inoculation to *Caryopteris*
clandonensis seedlings.

Partially - purified preparations of CCLV2 from infected *C. quinoa* were mechanically inoculated to 6 *Caryopteris clandonensis* seedlings at the 4 leaf stage. After inoculation all seedlings were kept in a glasshouse at 15°-25°C. to look for symptom development together with four apparently healthy seedlings for comparison. After six weeks, there were no symptoms on inoculated leaves, but four of the inoculated plants showed a reduction in growth compared with the healthy plants. These plants also had very thin stems, and smaller narrow leaves with smooth margins (plate 34). The leaves from each plant were harvested for backtesting to *C. quinoa* seedlings. Extracts from all four plants with reduced growth produced symptoms on *C. quinoa* characteristic of SLRV, and this virus was confirmed in gel diffusion tests.

4.25 Establishment of *Caryopteris clandonensis*
in tissue culture.

Material for in vitro culture originated from a single parent, the *Caryopteris clandonensis* clone 13 plant infected with strawberry latent ringspot virus (SLRV) obtained from Long Ashton Research Station.

Plate 34 *C. clandonensis* plant (right) inoculated with partially-purified preparation of CCLV2. Note the reduction in growth, thin stems and smaller narrow leaves with smooth margin, compared with the uninoculated plant (left) of the same age.



Initial cultures were established using nodal segments (1.5-2 cm.in length) selected from the original bush during a period of active growth. The explants were surface sterilized after removal of leaves, as described in Chapter 2.17. Nodal stem segments were aseptically placed upright on woody plant medium (WPM) adjusted to pH 5.7 with 0.1 N sodium hydroxide containing 0.5 μ M of benzyladenine (BA). Cultures were kept at 25°C. in growth room, illuminated with 20W fluorescent tubes, giving a flux density of 30 μ E/m²/S and a 16-hour photoperiod.

About 15% of cultures showed contamination with fungi or bacteria and had to be discarded. After 2-4 weeks in initiation medium, most lateral buds had developed into elongate shoots, and a creamy gall-like callus developed at the basal end of each segment which remained undifferentiated. About 75% of the explants proliferated.

Every 4 weeks, in vitro derived shoots 1.5-3.0 cm.long, and shoot tips (0.5 cm.) were excised and transferred to fresh culture jars. For this the shoots were cut into small segments about 1.0-1.5 cm. in length, each with one or two nodes. They were then placed, without additional

sterilisation treatment, on fresh medium in order to produce explants for virus elimination experiments.

4.25 a) Rooting in vivo

Rooting in vivo may be attempted directly on individual shoots produced in vitro by treating the shoots as micro-softwood cuttings (Deberghe and Maene, 1981). In this experiment, an attempt was made to root shoot tip explants about 3-4cm. in length. The basal ends of shoots were dipped in IBA solution at 0.5g/l for periods of about 1 min. before being placed in a (1:1) mixture of peat and sand compost, in 20 cm. pots, which were covered with clear polyethylene film to maintain humidity. Plantlets were maintained in a growth chamber at 25 °C. and illuminated from cool-white fluorescent lamps for 16 hr, daily at a light wavelength of 400-700 nm. The plants were sprayed with water once every day during the first 2 weeks, and every other day during the following 2 weeks. The above regime, however, was not successful as after two weeks the plantlets started to wilt and defoliate. Only a few root primordia were observed to have differentiated in the pith region of the stem. These primordia remained in the pith without further growth. Within 4 weeks, all of the plantlets died.

4.26 Attempt to eliminate SLRV from *Caryopteris*
clandonensis grown in vitro.

In the present study, both, thermotherapy and chemotherapy, in combination with tissue culture were used.

4.26 a) Heat therapy

Thermotherapy applied in a variety of ways has proved successful for the production of virus-free material (Nyland and Coheen, 1969) including several woody hosts (Hollings, 1965). High temperature treatment of infected plants frequently produces virus-free shoot tips which can be propagated as cuttings (Welsh and Nyland, 1965) or heat treatment may be followed by tissue culture of the vegetative meristem to produce virus-free plants (Hollings, 1965). Sweet et al. (1979) used heat therapy, meristem tip culture and the two combined to eliminate ArMV and RRV from *Daphne* "Somerset", RRV from *Daphne* "Somerset Gold Edge" and CMV from *D. odora*. These authors reported that the combined treatment was more effective than either alone for elimination of the two nepoviruses. Cooper and Walkey (1978) reported that cherry leaf roll virus in tissue cultures of *Nicotiana rustica* was permanently eradicated from most plants after 20

days incubation at 32°C. and from all plants after 7 days incubation at 40°C. These results confirmed earlier reports by Walkey (1976) that temperature and length of exposure were critical.

In this study, tissue cultures of infected *Caryopteris clandonensis* clone 13 were exposed to temperatures of c. 32°C. for continuous periods of 1-8 weeks.

Individual cultures were established by aseptically removing shoot tips about 2.5 cm. in length and dividing into a shoot tip and two nodal segments; each group of three explants was placed in a culture jar with fresh WPM. The culture jars were incubated in constant temperature cabinets at a temperature 32°C. with 30,000 lux, daylight fluorescent tubes for 16 hr.daily. The control cultures were put at 25°C. under the same light regime. Every 2 weeks, 15 jars from each treatment were taken, and shoot tips about 0.3-0.5 cm. in length were removed, and put into 3.5 cm. Petri dishes with damp filter paper. They were then kept in a deep freezer at -20°C. until being tested for virus.

Indexing for SLRV was done by grinding shoot tips in chilled watch glasses with a few drops of cold 0.1 M phosphate buffer pH 7.5, and

after adding a pinch of celite rubbing the extract on young leaves of *C. quinoa* indicator seedlings. A proportion of shoot tips from the heat treatment were cultured on fresh medium and transferred to the growth cabinet at 25°C. for observation of explants after thermotherapy. The results are present in Table 32.

Table 32 Effect of length of exposure at 32°C. on elimination of SLRV from *Caryopteris* explants

<u>Time in culture</u>	<u>% plants free of SLRV</u>
<u>no.infected /no.plants tested</u>	
2 weeks / (12/40)	70
4 weeks / (3/34)	91
6 weeks / (2/40)	97
8 weeks / (0/30)	100
Control(at 25 °C.)(30/30)	0

The shoot tip infectivity assays after only at 2 weeks treatment at 32° C. gave an apparently high percentage of virus- free plants. As the incubation time at 32 °C. increased so did the proportion of virus free explants. However, after 4 weeks the treated plants showed an unusual degree of shoot proliferation. The explants produced more shoots than the control at 25°C. and shoots had

increased internode length. The leaves were also smaller (plate 35). However, after the plantlets had been subcultured to fresh medium and transferred to 25°C., the excessive growth was reversed and plantlets developed normally.

To confirm that virus had been eliminated from the treated cultures, frozen samples were indexed by ELISA. An SLRV antiserum with a titer of 1:128 was obtained from rabbits immunized with purified SLRV and its gamma globulin was purified and conjugated with alkaline phosphatase (Chapter 2.15 c). Direct ELISA (DAS) was performed according to the procedures of Clark and Adams(1977). Leaf tissue was homogenized 1:10 (w/v) in 0.01 M phosphate buffer containing 0.15 M sodium chloride pH7.4, 0.05% (v/v) Tween 20, 2% (w/v) polyvinylpyrrolidone (Mr 40,000) and 0.2% (w/v) ovalbumin. Alkaline phosphatase - labelled - globulin was used at about 5 μ g/ml and p-nitrophenyl phosphate substrate was used at 1 mg/ml concentration.

The optical density of the enzyme-substrate reaction in each well, was measured spectrophotometrically at 410 nm. The infected leaf tissues from plants at 25°C. were used as positive controls and readings were taken one hour after

Plate 35 Unusual degree of shoot proliferation on *C. clandonensis* explant (right) grown in vitro at 32°C. for 4 weeks. Explant on left grown at 25°C.



addition of substrate. The plate was blanked against empty wells.

The absorbance range for sample from healthy *Caryopteris* grown in vitro was 0.09-0.15, the range for positive control sample was 0.6-0.85. Because of the variability in absorbance of healthy extract anything above 0.15 absorbance was regarded as positive. The same samples which were negative by infectivity assay were also found to be negative by ELISA.

This result confirmed that attempts to eliminate SLRV from *Caryopteris clandonensis* using heat treatment and tissue culture were successful.

4.26 b) Chemotherapy

Some viruses are difficult to eliminate by conventional means, such as heat therapy or meristem tip culture, and a number of authors have investigated the use of therapeutic chemicals, although none have found widespread practical application (Matthews, 1981). One of these compounds, ribavirin (syn. Virazole, 1- β -D - ribofuranosyl - 1,2,4-triazole-3-carboxamide), a synthetic nucleoside analogue, has a broad spectrum of antiviral activity against animal viruses

(Sidwell et al., 1972) and plant viruses in both herbaceous(Lerch, 1977., Schuster, 1979; Hansen, 1979; Hansen and Green, 1982) and woody hosts (Hansen, 1984). Moreover, incorporation of ribavirin into tissue culture media appears to suppress virus multiplication in plant tissues grown in vitro (Shepard, 1977., Simpkins et al., 1981). Ribavirin seems to act by inhibiting RNA and DNA synthesis rather than by directly affecting the virions (Smith, 1980), although in animal cells at least it may have several modes of action (Sim and McCullagh, 1985).

Among the other widely tested antiviral chemicals is amantadine, which has been shown to be effective in eliminating chrysanthemum stunt viroid from about 10% of chrysanthemum plants regenerated from shoot apices (Horst and Cohen, 1980). The action of this compound on viruses seems to be to prevent expression of the virus genome shortly after infection (Hoffmann et al., 1965., Skehel et al., 1977), possibly during uncoating. As viroids have no coat protein, amantadine must have other mode of action.

In the present study the effects of ribavirin and amantadine on SLRV-infected *Caryopteris clandonensis* were tested.

Concentrations of ribavirin of 1, 10 and 100 mg/l or amantadine at 25, 50 and 75 mg/l were incorporated into the tissue culture medium before it was autoclaved. Shoot tips were transferred from the stock culture to these media with different concentrations of chemical. All cultures, with or without chemical, were maintained at 25 °C. and grown under 30,000 lux daylight fluorescent tubes with a 16 hr. photoperiod. After 1 month, the shoot tips were collected from each treatment and kept frozen at -20°C until infectivity was assayed as described in Section 4.26 a.

Amantadine supplemented tissue culture media on which infected shoot tips were grown provided 4%, 11% and 30% virus free plants in three different concentrations (Table 33). No phytotoxicity was detected in plants grown on amantadine supplemented media within a month.

Table 33 Percentage of *Caryopteris* plants free of SLRV after growth on tissue media supplemented with antiviral compounds.

Chemical Concentration mg/l	% free of virus	Phytotoxicity
Ribavirin 1 mg/l(31/34)	9	0
Ribavirin 10 mg/l(26/31)	16	+
Ribavirin 100 mg/l(11/23)	52	++
Amantadine 25 mg/l(26/27)	4	0
Amantadine 50 mg/l(24/27)	11	0
Amantadine 75 mg/l(19/27)	30	0
Control (Untreated) (30/30)	0	0

The plants on ribavirin generally gave a higher proportion of apparently virus-free material up to a maximum of 52% (Table 33). Phytotoxicity was observed in plantlets grown on media with ribavirin at 10 or 100 mg/l. The plants were very stunted in growth and had a scorch on the lower leaves even at 10 mg/l. (plate 36) Simpkins et al., (1981) found that ribavirin at concentrations of 10, 50 and 100 mg/l caused no damage to cultures of *N. rustica*, but was phytotoxic to chickweed at 50-100 mg/l. Shepard (1977) similarly reported that 10 and 100 mg/l ribavirin reduced growth rate and survival among calli. Perkins (1987) noted the phytotoxicity of ribavirin to several woody ornamentals grown in vitro.

Plate 36 *Caryopteris clandonensis* explants grown on medium with ribavirin at (from left to right) 0, 1, 10 and 100 mg/l.



4.27 Discussion

Several lines of evidence suggested that CCLV2 belonged to the nepovirus group. For example, the wide experimental host range and the development of ringspot lesions on certain hosts were characteristic of nepoviruses (Murant, 1981). Another group - the cucumovirus - which have wide host ranges, and isometric particles like CCLV2, do not produce a systemic reaction in *Chenopodium quinoa* which was highly susceptible to CCLV2.

The positive soil bait test, which suggested that the causal agent was soil-borne, further indicated that CCLV2 belonged to the nepovirus or possibly tobacco necrosis virus (TNV) group. The host reactions of CCLV2, however, were quite distinct from those reported for TNV (Uyemoto, 1981). Many other characteristics such as moderate stability in vitro, high levels of seed transmission, analysis of dsRNA, and the presence in the electron microscope of tubules in extracts of infected tissues, were also consistent with a nepovirus diagnosis.

Because of a considerable overlap between different nepoviruses and between some strains of the same virus, symptom expression or physical properties of the virus are not a reliable

means of identifying individual nepoviruses. However, the high frequency of symptomless test plants, especially among *Nicotiana spp.*, and the known UK distribution of these viruses in woody ornamentals (Murant, 1981), suggested that CCLV2 might be strawberry latent ringspot virus (SLRV), or possibly arabis mosaic virus (ArMV). Previous isolates of SLRV from horse chesnut (Perkins, 1987) and rose (Hicks, 1979) induced similar symptoms to CCLV2 in herbaceous test plants. SLRV also infected most of the 126 hosts listed by Schmelzer (1969) without showing symptoms. Arabis mosaic virus isolates from woody ornamentals tend to show more severe symptoms than those seen with CCLV2, especially in *N. tabacum* (Hicks, 1979; Perkins, 1987). The dilution end-point, thermal inactivation point, and longevity in vitro of CCLV2 were similar to those reported for SLRV from other hosts (Lister, 1964; Walkey and Mitchell, 1969; Allen et al, 1970; Murant, 1974; Hicks, 1979; Cooper, 1981; Perkins, 1987).

Confirmation that CCLV2 was an isolate of SLRV was obtained by serology. Additional evidence was provided by coat protein analysis. Unlike most nepoviruses which have an Mr of about 55,000, CCLV2 had two coat protein components of Mr

27,500 and 42,000. In the Mr of its coat protein, SLRV is similar to the comoviruses (Matthews, 1981), although, as a group, the latter usually have a fairly narrow host range (Stace-Smith, 1981). Analysis of dsRNA isolated from infected *C. quinoa* produced 3 species of RNA with Mr similar to those reported for SLRV (Mayo et al., 1982). The observation that RNA 3, presumably SLRV satellite RNA, was lost after the helper SLRV had passed through seed should be explained further. Possibly the satellite RNA was susceptible to inactivation by the conditions inside floral meristems or developing seeds.

The apparently isolated occurrence of CCLV2 at Long Ashton, with one plant of one clone affected, indicated that infection was not widespread in *Caryopteris clandonensis* either at Long Ashton or at the original contributing nursery. Infection was confirmed as soil-borne at the Long Ashton site but it is not known if the resident vector population simply acquired SLRV from an already infected *Caryopteris*, or was responsible for initiating the infection. Whatever the route of infection, the virulence of the isolate was clearly demonstrated by inoculation of seedling *C. clandonensis*. Within six months, the mechanically inoculated plants were reduced in vigour and showed

the leaf narrowing characteristic of the disease in the original bush. Further studies are need to establish whether this was a new 'pathotype' of SLRV or whether it simply reflected the broad natural host range shown by many field isolates of SLRV (Murant, 1981).

The initiation and establishment of *C. clandonensis* in tissue culture was apparently the first report for this genus. Limited attempts to obtain rooting, however, were not successful although a few root initials were produced. The level and type of plant growth regulator, the type of micro-cutting, and the temperature are all factors that can influence root initiation and development, and should be tested, in future, with *C. clandonensis*. The production of rooted plantlets is essential if the potential benefits from virus elimination are to be exploited in clonal improvement programmes.

The attempts at virus elimination in tissue culture indicated that thermotherapy was a promising technique. Some damage to the cultured tissues, however, was observed although when grown at lower temperatures the explants recovered. To reduce the likelihood of heat damage it may be worthwhile testing lower temperatures for longer

periods to see if the same levels of elimination can be achieved. Similar temperatures to those used for CCLV2 elimination have been used for other nepoviruses such as cherry leaf roll in *Nicotiana rustica* (Cooper and Walkey, 1978).

The two antiviral compounds tested were less effective at virus elimination than thermotherapy. Reduction in growth and scorching were observed at the two highest concentrations of ribavirin. Similar phytotoxicity with ribavirin was reported by Simpkins et al (1981), and Cassells and Long (1983) in *Nicotiana*. Ribavirin, a nucleoside analogue, may act by inhibiting viral RNA and DNA synthesis. Effects on host nucleic acid metabolism probably explains the cytotoxicity sometimes associated with its use. The effectiveness of ribavirin does vary with the virus and host, but several authors reported increased frequency of production of virus-free plants in tissue cultures (Cassells and Long, 1980; Simpkins et al., 1981; Klein and Livingstone, 1983).

In the one experiment in which comparisons were made, there were no marked differences in the detection of SLRV in tissue cultured material by infectivity assay on *Chenopodium quinoa* or by ELISA. It is possible,

however, that some of the borderline positives were high negatives. It would be important to retest material classified as negative before it can be considered as virus-free, because low levels of virus may escape detection.

CHAPTER 5 : VIRUS INFECTION OF *SENECIO GREYI*

Senecio is one of the largest genera of flowering plants and comprises over 1,000 species, belonging to Compositae family (Bean, 1980). The generic name, which was used by Pliny, is derived from the latin senex, an old man, and refers to the usually grey hair-like pappus of the seeds.

The *Senecio* genus has alternate leaves and the flowers are crowded in heads. The florets are usually of two kinds, those in the centre of the head, of tubular shape and known as "disk" florets and those of the circumference, tongue-shaped, rachating, and known as "ray" florets.

There are several reports of virus infection in *Senecio spp.* Taylor and Thomas (1968) reported seed transmission of arabis mosaic through *Senecio vulgaris*. In addition, *Senecio vulgaris* was found to be one of a number of symptomless weed plant sources of CMV in lettuce fields, and believed to be the major overwintering source of inoculum for lettuce (Tomlinson et al., 1970). Rupple and Duffus (1971) also demonstrated mechanical transmission of beet yellow vein virus to *Senecio vulgaris*.

In India, a mosaic disease of *S. cruentus* was detected by Singh et al. (1975). A sap transmissible virus was isolated from diseased plants and the virus was designated " Senecio mosaic virus". Its host range was restricted to *Senecio*, *Nicotiana*, spinach and *Chenopodium*, although the agent was not fully characterised. In the same year, a strain of CMV was isolated from the perennial ornamental *Senecio kaempferi* which was reported to play a part in the conservation of the virus from year to year (Ragozzino and Stefanis, 1975). The disease symptoms were reproduced on *S. kaempferi* seedlings in the glasshouse inoculated with the CMV isolate.

Furthermore, Hartleb et al. (1978) reported that *S. vulgaris* was an overwintering source of beet mild yellowing virus(BMYV). Morvan and Chastelliere (1982) suggested that *S. sylvaticus* was the best test plant for isolating plum pox virus (PPV) from woody sources and Credi (1985) demonstrated that beet western yellow virus (BWYV) was transmitted consistently by *Myzus persicae* to *S. vulgaris*.

No previous report on virus infection in *S. greyi* was found.

During a routine survey of shrubs for virus infection, two isolates were obtained from *Senecio greyi* growing in flower borders in two different parts of the country. The first isolate (SV1) was obtained from a *S. greyi* bush in Norfolk with occasional mild yellow chlorotic spots and mottle on the leaves and slight distortion on the margins of the young leaves. Only a small proportion of shoots showed symptoms at any one time. There were no obvious effects of infection on vigour when compared to adjacent uninfected bushes.

The second isolate (SV2) was obtained from a *S. greyi* plant, grown on the campus of Bath University. The plant was one of several in the flower border infected apparently with the same virus. All plants were of low vigour compared to an apparently virus-free bush in the same bed; one stunted plant had died (plate 37). The leaves on diseased plant were slightly reduced in size and had "raised" dark-green areas and uneven leaf margins especially on young leaves (plate 38). Infected leaves were paler than uninfected leaves and sometimes showed chlorotic spots and mottle. This symptom was observed clearly in the new growth produced in spring or early summer.

Plate 37 Reduced vigour in *Senecio greyi* infected (+) with SV2



Plate 38 *Senecio greyi* leaves infected with SV2 showed reduction in size, uneven leaf margins and chlorotic spots.



5.01 Isolation of virus from diseased plants

Both viruses, SV1 from Norfolk and SV2 from Bath, were readily isolated from young leaves with or without symptoms especially in spring and summer, but also from plants held in the glasshouse during winter. The leaves were ground 1:3 (w/v) in 0.1 M phosphate extraction buffer pH 7.5 without additives. After addition of a little celite, the extract was inoculated to a range of test plants including *Chenopodium* spp., *Nicotiana* spp., *Cucumis sativus* and *Phaseolus vulgaris*. *Chenopodium amaranticolor* and *C. quinoa* were usually the plants species to develop symptom in the first subculture from *Senecio*. Symptom on *Chenopodium* spp. were chlorotic and/or necrotic local lesions which developed within about 5 days after inoculation.

5.02 Herbaceous host range of SV1 and SV2

The herbaceous host range of SV1 and SV2 isolates were compared using single lesion isolates established from cultures in *C. quinoa*. Both inocula were prepared by grinding inoculated leaves of infected *C. quinoa* 1:4 (w/v) in 0.05 M phosphate extraction buffer pH 7.5. The host ranges were determined twice and consistent symptoms are presented in Table 34.

Table 34 Symptoms induced on herbaceous hosts by
two isolates from *Senecio greyi*

Host Plant	SV1	SV2
<u>Chenopodium amaranticolor</u>	NL/O	NL/O
<u>C. foetidum</u>	CL-NL/O	CL-NL/O
<u>C. murale</u>	NL/O	NL/O
<u>C. quinoa</u>	CL-NL/O	CL-NL/O
<u>Cucumis sativus</u>		
cv. "Parisienne Pickling"	CL/CM,D	CL/CM,D
<u>Datura stramonium</u>		
var. "tatula"	CL/SI	CL/SI
<u>Gomphrena globosa</u>	NL/SI	NL/SI
<u>Lycopersicon esculentum</u>		
cv. "Moneymaker"	NL/CM	NL/CM
<u>Nicotiana clevelandii</u>	CL/CM	CL/CM
<u>N. debneyi</u>	CL/CM,D	CL/CM,D
<u>N. glutinosa</u>	CL/CM	NR/CM
<u>N. megalosiphon</u>	NL/VN-Dth	NL/VN-Dth
<u>N. tabacum</u> cvs. "Xanthi"	CL-RS/M	CL/CM
<u>N. tabacum</u> cvs. "White Burley"	CL/M	CL/M
<u>Phaseolus vulgaris</u> cv. "The Prince"	NL/O	NL/O

Abbreviation : local reactions / systemic symptom

C = chlorotic or chlorosis	VN = vein-necrosis
N = necrotic	D = distortion
SI = symptomless infection	L = local lesion
M = mottle	Dth= death
RS = ringspots	O = no infection

Symptoms produced by both SV1 and SV2 isolates were similar in many hosts.

Thus, both isolates infected *Chenopodium spp.* with the production of local lesions (plate 39), but no systemic spread. Characteristically, *C. amaranticolor* produced small necrotic pin point lesions within 3 to 4 days of inoculation with both isolates. About 5-6 days after inoculation *C. quinoa* developed either large yellow local lesions about 1-2 mm. in diameter with irregular, etched, brown borders (SV2) or diffuse yellow chlorotic spot 2-3 mm. in diam. (SV1), which became white and necrotic in the centre with a brown ring and chlorotic halo after 7-10 days (plate 39 a). On *C. murale* both SV1 and SV2 produced white necrotic spots on inoculated leaves (plate 39 b).

Most *Nicotiana spp.* showed chlorotic spots or rings on inoculated leaves (plate 40) and a systemic mosaic or mottle with both isolates on *N. glutinosa*. However, SV2 produced necrotic ringspots on inoculated leaves rather than chlorotic rings, lines and spots (SV1)(plate 41).

On French bean (plate 42) white necrotic lesions were detected in winter and occasionally in summer, with both isolates.

Plate 39 Local necrotic spots with chlorotic halos on *C. quinoa* (a), local chlorotic/necrotic lesions on *C. murale* (b) infected with SV1.

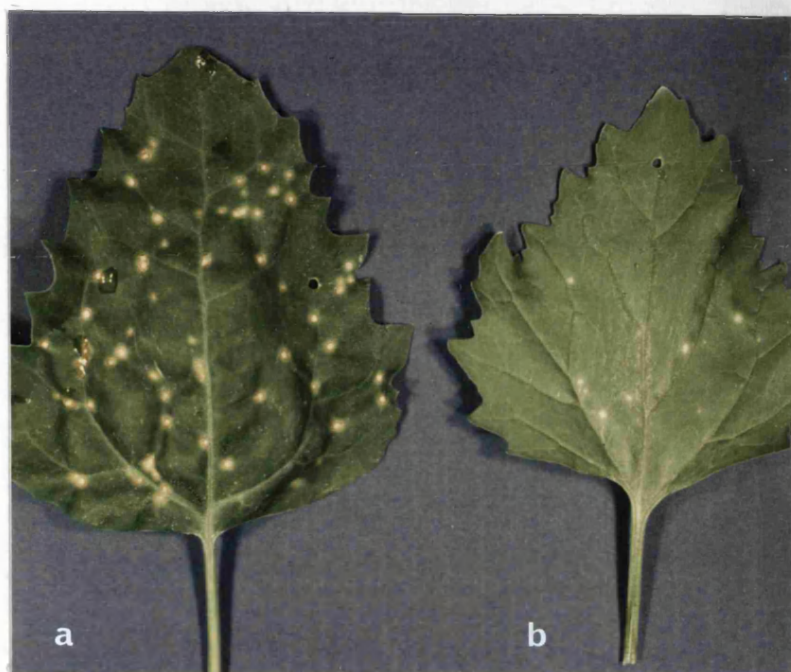


Plate 40 Chlorotic rings and spots on inoculated leaf of *N. rustica* infected with SV1.



Plate 41 Chlorotic local lesions on inoculated leaves of *N. glutinosa* infected with SV1.



Plate 42 White necrotic lesion on inoculated leaf of *Phaseolus vulgaris* infected with SV2.



5.03 In vitro properties of SV1 and SV2 *Senecio greyi*

Sap from these tests were prepared by extracting systemically-infected leaves of "Xanthi" tobacco in 0.05 M phosphate buffer pH 7.5. Samples were assayed for infectivity on *C. quinoa*.

5.03 a) Dilution end point

The DEP of SV1 and SV2 were shown to be 10^{-3} to 10^{-4} .

5.03 b) Thermal inactivation point

The SV1 isolate lost infectivity between 50°-60°C. while SV2 was between 65°-70°C.

5.03 c) Longevity in vitro

Sap samples infected with SV1 lost infectivity within 24 hours at room temperature, whereas, SV2 retained infectivity for 2 to 4 days. However, sap samples in the deep freeze remained infective after 1 1/2 years, the longest period tested, with both isolates.

5.04 Serology : Identification of SV1 using ELISA technique

Test plant reactions indicated that both SV1 and SV2 might be cucumber mosaic virus.

The experiment was performed based on the method of Lommel et al. (1982) see Section 2.15 c. and 4.05.

An ELISA test (4.05) was set up using an antiserum, raised against CMV-*Connelina nudiflora* and obtained from American Type Culture Collection.

Infected *Senecio greyi* leaves with SV1 was extracted in antigen buffer at a dilution of 1:4, 1:40, 1:400 and 1: 4000. Sap of SV1-infected *N. tabacum* "Xanthi", at the same dilution, was prepared as a control, and also healthy sap of both plants were included in the test.

Enzyme-conjugated protein A was applied at a dilution of 1:5000 and antiserum was used at 1:3000. All samples were tested in duplicate and the incubation time for substrate was overnight at 4°C.

The mean absorbance values (410 nm) for the test are presented in Table 35 .

Table 35 Comparison of the mean absorbance of SV1 infected *Senecio greyi* and *N. tabacum* "Xanthi"

Host Plant	Mean A 410 nm	
	Infected	Healthy
<i>Senecio greyi</i> ;		
1:4	0.994	0.199
1:40	1.036	0.268
1:400	0.739	0.138
1:4000	0.280	0.112
<i>N. tabacum</i> "Xanthi" ;		
1:4	>2.0	0.053
1:40	>2.0	0.069
1:400	1.432	0.060
1:4000	1.207	0.055

From the results, the test confirmed that SV1 was CMV, because the mean absorbance for infected extracts was greater than twice that of the healthy controls at all sap dilutions. The results indicated that at equivalent dilutions the herbaceous host (*N. tabacum*) contained much higher concentration of virus than *Senecio*. The healthy background values were also higher for *Senecio* extracts. The results suggested that the protein A ELISA had potential for the diagnosis of CMV in *Senecio* in field infections.

5.05 Double-stranded RNA analysis

In a preliminary experiment, dsRNA was extracted (Section 2.14) from about 15 g. of "Xanthi" tobacco leaves infected with SV1 or SV2. Samples equivalent to 15 g. tissue were electrophoresed for 4 hr. on 6% polyacrylamide gels and stained with ethidium bromide.

To provide further support for this identification, the band patterns produced by dsRNA's extracted from SV1-and-SV2 infected *N. tabacum* "Xanthi" plants, were compared with band patterns of dsRNA extracted from the same cultivar infected with CMV 'W' strain (from lettuce) on a minislab gel with 0.6% agarose. The samples of dsRNA, were run at 20 volts for 3 1/2 hr. Band patterns produced on the gels by all 3 isolates were similar, with the 4 "major" bands and the satellite moving the same distance in the gel. The presence of an additional "minor" dsRNA between dsRNA segments 2 and 3 with 'W' strain CMV but not other isolates may be due to higher concentration of dsRNA with the 'W' strain. In a later study, in which polyacrylamide gels were heavily loaded with dsRNA from SV1 or CMV-W infected tobacco, both isolates produced the minor band between dsRNA 2 and 3, but in addition strain 'W' produced a sixth band just below RNA 5 (plate

43). The Mr estimates for dsRNA for the three isolates were 1.99×10^6 (RNA 1), 1.81×10^6 (RNA 2), 1.25×10^6 (RNA 3), 0.62×10^6 (RNA 4), 0.46×10^6 (RNA 5) and 0.34×10^6 (satellite).

Both SV1 and SV2 produced identical dsRNA pattern on the gels and at least six bands were visible. The first four bands probably represented the double-stranded replicative form of genomic RNA, reported for CMV (Bar-Joseph et al., 1983; Morris et al., 1983). The fifth band was supernumerary, but is sometimes reported with CMV (Dodds, et al., 1984). Possibly this band was a sub genomic element of a longer dsRNA. In addition, the sixth low Mr segment was probably the dsRNA of the satellite found with many isolates of CMV (Kaper and Waterworth, 1981). The matching profiles of SV1 and SV2 suggested that SV2 also was CMV.

In another experiment, attempts were made, firstly, to detect CMV dsRNA in leaves of *S. greyi* infected with SV2, and secondly, to ascertain, by comparison of the same isolate in *N. tabacum* "Xanthi", whether the satellite was present with CMV in *Senecio* leaf tissue. Parallel extractions of dsRNA from *Senecio* were done. The results (plate 44) showed that it was possible to detect dsRNA components with direct extraction

Plate 43 DsRNA patterns on 0.6% agarose gel extracted from *N. tabacum* "Xanthi" infected with :-
(a) SV1 (b) SV2 (c) CMV "W" (lettuce isolate)
and lambda DNA digested with (d) Hind III
(e) Hind III double digest

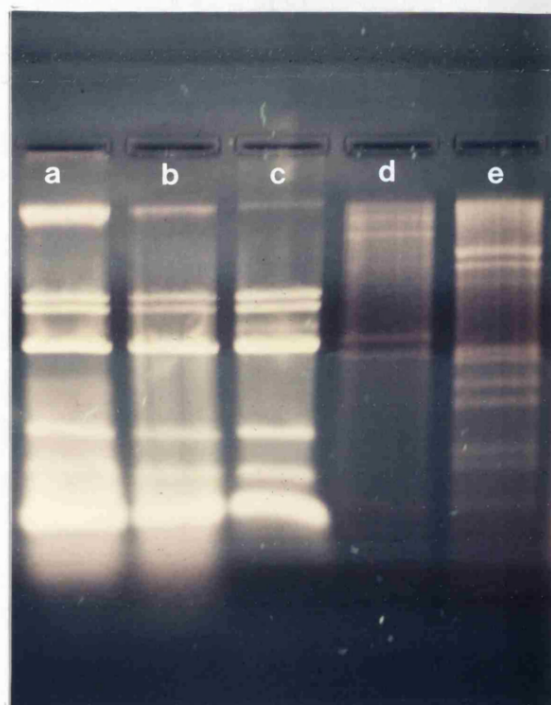
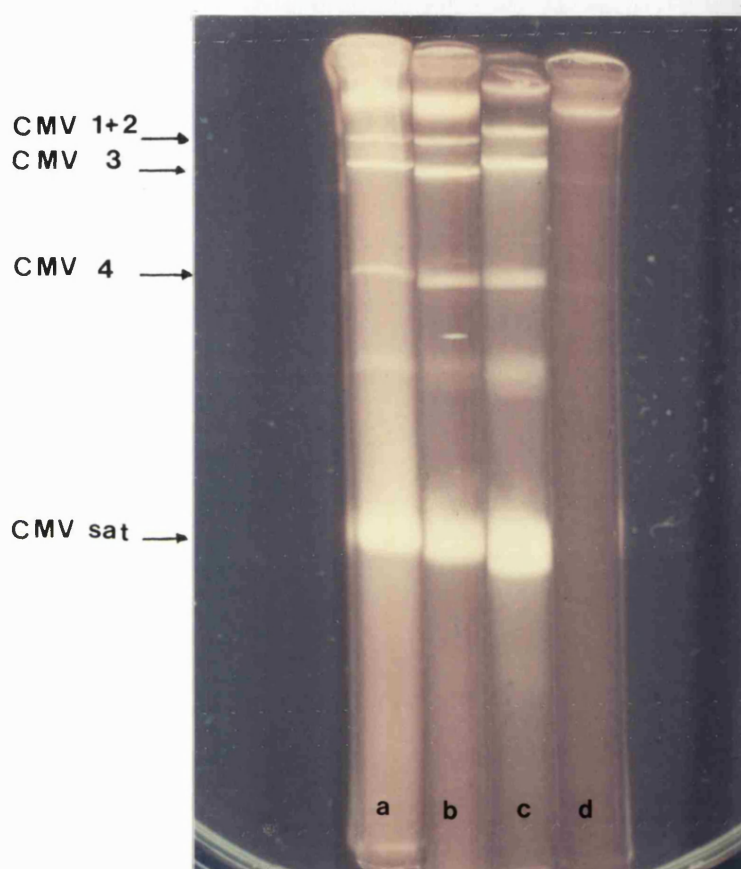


Plate 44 Ethidium bromide stained polyacrylamide gels showing dsRNA extracted from *N. tabacum* "Xanthi" infected with (a) CMV "W" (lettuce isolate) (b) CCLV1 (*Caryopteris* isolate) (c) SV2 and (d) SV2 extracted from *Senecio greyi*.



from woody host leaf tissues, but that for *Senecio*, at least, concentrations were low when compared to dsRNA extracted from lower weights of herbaceous host tissue.

After electrophoresis for 4 hr, it was clearly shown (plate 44) that tobacco plants infected with CMV isolates from lettuce, *Caryopteris* and *Senecio* supported the replication of the satellite RNA species. In contrast, no satellite species was detected in dsRNA preparations from *Senecio*. Either this RNA was not present, or its replication was suppressed below the limits of detection (estimated to be at least 100 ng/band).

5.06 Tissue culture of *Senecio greyi*

Attempts to establish infected *Senecio greyi in vitro* were unsuccessful due to heavy contamination of the parent material with systemic pathogens.

5.07 Discussion

The two *Senecio greyi* bushes from which the two isolates were obtained showed different symptom expressions. The Norfolk plant showed mild foliar symptoms with no apparent reduction in vigour, while the Bath plant had more

severe foliar symptoms with conspicuously reduced vigour. The variation in field symptoms, however, were seldom correlated with differences in other properties. Slight differences in symptom expression in some hosts (*N. glutinosa*, *C. quinoa*) were, nevertheless, observed and SV2 had a significantly higher TIP, than SV1, and was more stable in LIV tests. In these respects, SV2 resembled an isolate of CMV from *Rubus* (Jones, 1976), and the isolates from *Buddleia* and *Lonicera* (Perkins, 1987). The TIP (50°-55° C) of SV1 was lower than that for many isolates of CMV (Gibbs and Harrison, 1970; Francki and Hatta, 1980).

Analysis of dsRNA extracted from tobacco infected with either SV1 or SV2 showed the same banding patterns, suggesting a close relationship between the two isolates. Dodds et al (1985) found that two strains of CMV which differed in symptom intensity in tobacco, tomato and squash could be separable by the electrophoretic mobilities of dsRNAs 1 and 2.

Both SV1 and SV2 accumulated large amounts of satellite dsRNA in tobacco. In contrast, satellite dsRNA was not detected in dsRNA samples from *Senecio* infected with SV2. The possibility that

the absence of satellite RNA in the Bath shrub (SV2) contributed to the greater disease severity, compared to the Norfolk plant (SV1), should be explored further. It is as likely, however, that the symptom differences were due to factors other than intrinsic differences between the two isolates.

Both the isolates from *Senecio* differed in host range or reaction from some of the more commonly reported strains of CMV (Gibbs and Harrison, 1970; Kaper and Waterworth, 1981), as well as isolates from *Buddleia* and *Lonicera* (Perkins, 1987). Thus, these isolates typically produced 'fern-leaf' symptoms in tomato, whereas the *Senecio* isolates did not. However, relatively few of the strains encountered by Francki and Hatta (1980) in Australia, produced the classical fern leaf symptom although many produced mosaics of varying severity. The Scottish isolate from *Rubus* (Jones, 1976) produced no symptoms in tomato. Francki and Hatta (1980) also noted considerable variation in lesion type in *C. quinoa* and *C. amaranticolor* which was similar to the variation found between SV1 and SV2 in these hosts.

It is possible that both *Senecio* plants were infected at the same, or different, nursery source, through dissemination of infected,

vegetatively propagated material. However, the ready transmission of CMV by many aphid species, including the widespread *Myzus persicae* (Gibbs and Harrison, 1970), suggests the virus may have been picked up from aphids visiting *Senecio* from one of the many natural reservoirs of infection (Kaper and Waterworth, 1981).

CHAPTER 6 : OTHER VIRUS AND VIRUS-LIKE DISEASES OF SHRUBS.

During the survey of woody ornamental for virus infection conspicuous virus-like symptoms were seen on several shrubs. Limited attempts at isolation were made and, where possible, the viruses were identified and partially characterised.

PART A : *FORSYTHIA INTERMEDIA* ZABEL.

The genus *Forsythia* (Oleaceae), is a group of New World shrubs, of which three species are occasionally cultivated in botanical collections (Bean, 1980). The name of the shrub was given in honour of Charles Le Forestier, a French physician and naturalist. The leaves are deciduous, and opposite, the flowers small, greenish, without petals, and unisexual; the sexes are often on separate plants. They grow most soils, and are easily propagated by late summer cuttings.

In East Germany, Schmelzer (1962-63;1964) reported yellow net symptoms on leaves of *F. intermedia* infected by ArMV. Pathogenicity was demonstrated by mechanical inoculation of ArMV to cotyledons of *F. intermedia*. In USA, Tiangco and

Verny (1970) showed tobacco ringspot virus to be a cause of yellow net, although, this virus was also detected in symptomless *Forsythia* (Waterworth and Povish, 1972).

Raspberry ringspot virus (RRV) was detected in leaves of *F. sybaldii suspensa* Hort. and *F. intermedia* in U.K. with bright yellow ring patterns and vein yellow net (Cooper, 1979). In East Germany, tomato blackring, alfalfa mosaic and tobacco rattle viruses were isolated from *F. intermedia* (Schmelzer, 1959, 1962-63, 1974), and arabis mosaic virus from *F. europea* (Schmelzer, 1974) but their relationship to disease symptoms in *Forsythia* was not established.

6.01 Isolation of RRV from diseased *F. intermedia*.

The diseased plant was observed as part of a mixed hedge in the Victoria Park Botanical Garden, Bath. The plant had conspicuous leaf symptoms which included chlorotic rings and lines, vein yellowing and general chlorosis (plate 45). The causal agent was readily transmitted to a healthy 8 ft. tall *Forsythia intermedia* by flap grafting with leaf tissue from the infected shrub (see Chapter 3.08 b). Symptoms were visible in the new growth of the spring following grafting (September). The agent spread rapidly from the graft sites (3) so

that within 7-8 months after inoculation about 80% of the shoots had developed some symptoms.

In an attempt to isolate virus from the original shrub, young leaves (with symptoms) were homogenised in phosphate buffer (1:4 w/v) and after addition of a little celite, the extract inoculated to a range of test plants (see Section A). Clear local lesions developed within 7 days on *C. quinoa* and *C. amaranticolor*, and the latter used to establish a single isolate for host range studies. The symptom expression on test plants are presented in Table 36 .

Symptoms usually appeared on inoculated leaves within 5 to 7 days and on uninoculated leaves within 7 to 14 days. Symptoms in general consisted of chlorotic or necrotic lesions on inoculated leaves, and chlorotic mottle, ringspots and necrosis on systemically-infected leaves.

The wide host range, the presence of ringspots, and the "recovery" from symptoms in some *Nicotiana spp.* suggested that the *Forsythia* virus might be a nepovirus (Murant, 1981). Furthermore, the symptoms on the Bath shrub resembled those associated with infection by raspberry ringspot

virus in *Forsythia* (Cooper, 1979). The local lesion in *C. anaranticolor* without systemic spread (plate 46) and the systemic mottle and necrosis in *C. quinoa* (plate 47), which are both diagnostic reactions for RRV (Murant, 1981) supported this diagnosis.

6.02 Identification of the isolate

The *Forsythia* virus was identified using agar gel double-diffusion. Crude sap from infected *C. quinoa* leaves provided the antigen source which was tested against antisera (undiluted) to the nepoviruses ArMV (*Daphne* isolate), RRV (*Jasmine* isolate) and SLRV (parsnip isolate). Healthy *C. quinoa* sap was included as a control. After overnight incubation at room temperature a single precipitin line was formed between the antigen well and the well filled with RRV antiserum. No other precipitation lines developed and the result confirmed that the *Forsythia* isolate was RRV.

Plate 45 *F. intermedia* leaves naturally infected with RRV showing chlorotic ring and lines, and general chlorosis.



Plate 46 Chlorotic local lesions on *C. amaranticolor* inoculated with RRV.



Plate 47 Mottling on young leaves of *C. quinoa*
inoculated with RRV.

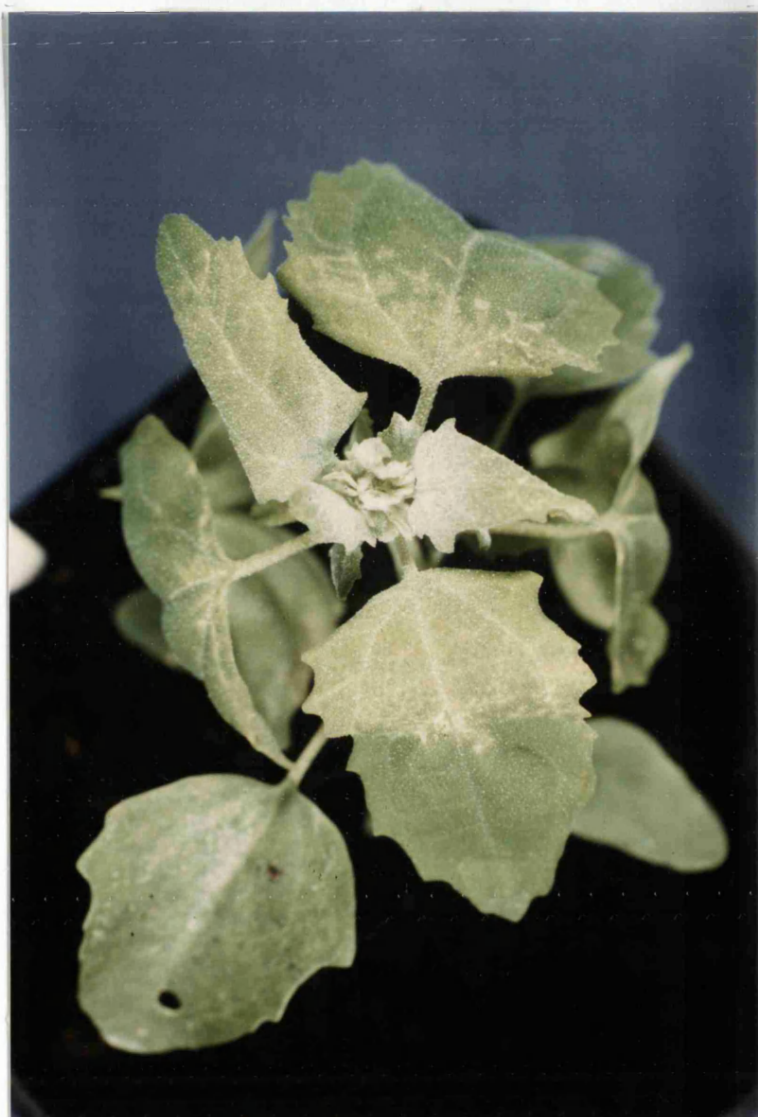


Table 36 Symptoms induced by the virus from *F. intermedia*

Host Plants	Symptom Expression
<i>Chenopodium album</i>	NL/N,D
<i>C. amaranticolor</i>	CL/O
<i>C. foetidum</i>	NL/N→DT
<i>C. murale</i>	NL/N
<i>C. quinoa</i>	CL/CM,D,N
<i>Cucumis sativus</i>	
cv. "Parisienne Pickling"	CL/CL,D
cv. "Marketer"	O/O
<i>Nicotiana clevelandii</i>	NS/RS,VN,D
<i>N. glutinosa</i>	NL/CM
<i>N. megalosiphon</i>	NL,NS/NS,VN
<i>N. rustica</i>	NRS/CL,RS,CM→R
<i>N. tabacum</i> "Xanthi"	NL,NRS/RS,CL,LP→R
<i>N. tabacum</i> "White Burley"	NL,NRS/CM,RS
<i>Phaseolus vulgaris</i>	
cv. "The Prince"	CL/CM,VY
<i>Pisum sativum</i>	
cv. "Montieth"	N/N→DT

Abbreviations : local reactions / systemic symptoms

C = chlorotic or chlorosis
N = necrotic or necrosis
L = lesions
M = mottle
LP= line patterns
R = recovery

VN = vein necrosis
D = distortion
O = no infection
RS = ringspots
VY = vein yellowing
DT = death

6.03 Purification of the virus

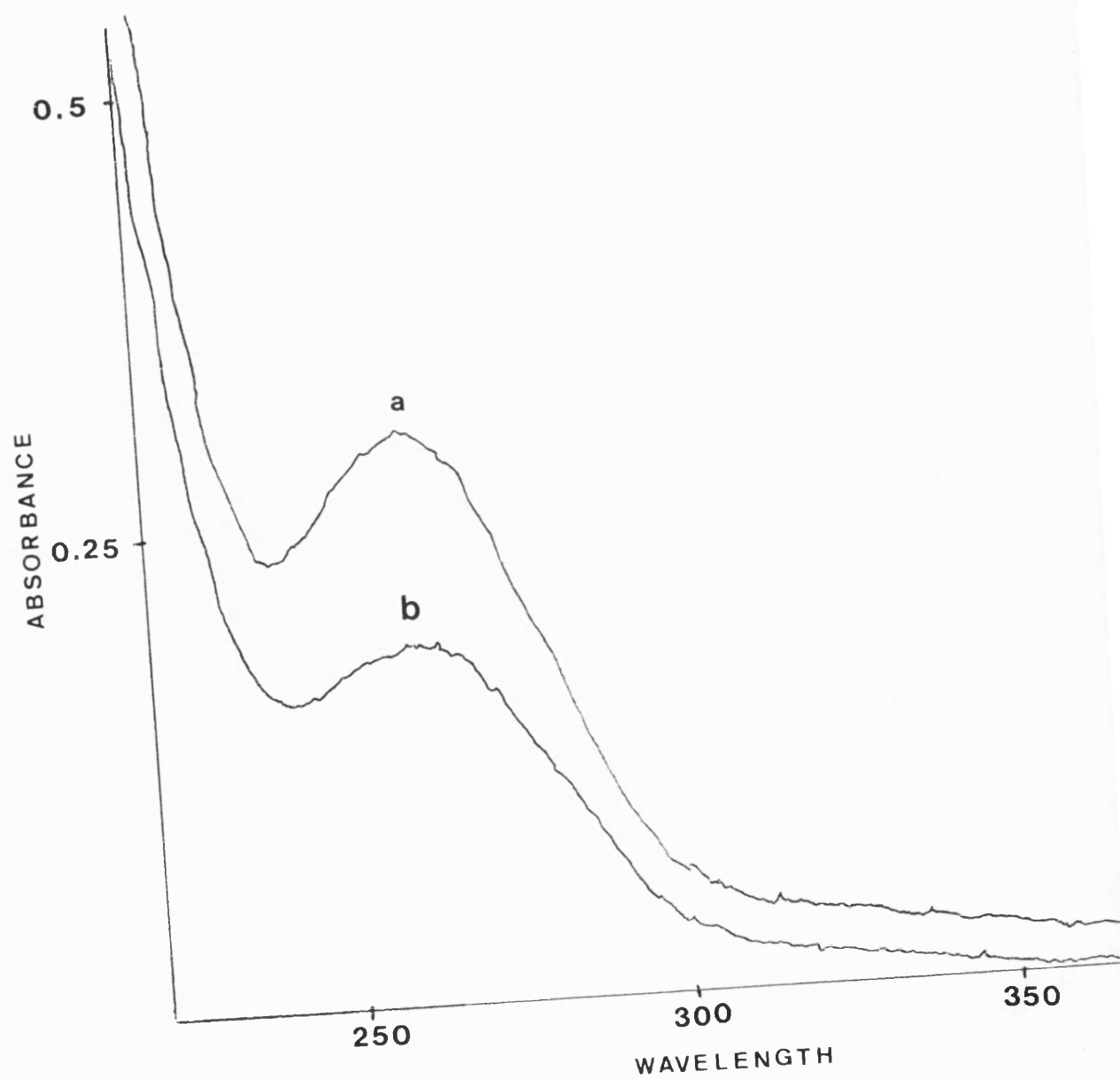
In a preliminary study, purification of *Forsythia* RRV was attempted based on a method recommended by CMI leaflet revised by Murrant (1981). Two hosts were compared as potential sources of virus : *N. rustica* and *N. tabacum* cv. "Xanthi".

One hundred grams of leaf tissue from each host was purified by blending for two minutes with one volume (100 ml) of phosphate buffer pH 7.8 containing 0.01 M sodium mercaptoacetate and 0.01 M EDTA, and one volume of chloroform. The extracts were clarified by low speed centrifugation at 5000 g for 15 min. and virus precipitated from the supernatant by addition of 100 g/l polyethylene glycol (PEG 6000) and 10 g/l NaCl. After the chemicals had dissolved, and the solution had stood for one hour at 4° C., the virus was pelleted by centrifugation for 25 min. at 10,000 g. The pellets were resuspended overnight at 4° C. in a total of 2 ml of 0.05 M phosphate buffer pH 7.8. After a brief bench centrifugation the U.V. data for each solution was determined at 1:100 dilution (Figure 14). For both *N. tabacum* "Xanthi" and *N. rustica* extracts a curve typical of nucleoprotein was obtained. The uncorrected A 260/280 ratio were 1.58 and 1.54, respectively. Using an extinction coefficient of 7

for RRV (Murant, 1978), the concentration of the sample from *N. tabacum* "Xanthi" purified was 4 mg/ml, while from *N. rustica* was 2.6 mg/ml. These were confirmed by infectivity results (5 replicates) as a number of lesions on *C. amaranticolor* leaves inoculated with 1:20 dilution of partially-purified preparation from *N. tabacum* "Xanthi" extract was 47, whereas from *N. rustica* was 21.

The results suggested that in this study *N. tabacum* "Xanthi" was the better source of RRV.

Figure 14 Absorption spectra of partially-purified preparation of RRV (1:100 dilution) from
(a) *N. tabacum* "Xanthi"
(b) *N. rustica*



PART B : SPIRAEA BUNALDA "ANTHONY WATERER"

Spiraea (Rosaceae) is a genus of hardy flowering shrubs known to be susceptible to infection with ArMV. The virus was isolated from *S. bunalda* Burren. with yellow vein-netting (Schmelzer, 1970), *S. douglasii* Hook. with vein-yellowing, and enations on leaves (Sweet and Campbell, 1975 a), and from symptomless *S. albiflora* (Mig.) Zab.(syn. *S. japonica*) L. var. alba) (Sweet, 1975 b). Sweet (1975 b) suggested that the occasional pink or white variegation on the leaves of *S. bunalda* cv. Anthony Waterer was non-pathogenic. Attempts to reproduce symptoms in *S. albiflora* seedlings by approach-grafting to *Chenopodium amaranticolor* plants infected with a *Spiraea* isolate of ArMV were unsuccessful, although seedlings grafted to ArMV-infected *Spiraea* became infected, but without producing symptoms (Sweet, 1975 b). Perkins (1987) failed to isolate a virus from cv. "Anthony Waterer" with variegation.

6.04 Isolation of ArMV from *S. bunalda* "Anthony Waterer"

Two plants of "Anthony Waterer" growing in a flower border at Bath University had shown vein-yellowing symptom, particularly on older leaves, and, occasionally, chlorotic mottle (plate

48). Plants were also slightly reduced in vigour compared to adjacent apparently healthy plants. Symptoms tended to be more severe late in the season although this year (1989), no symptoms have appeared at all.

To test for virus, inoculum was prepared by grinding young leaves (without symptoms) in phosphate buffer (1:4 w/v). The extract was inoculated to a range of host plants described in Section A. After 5-7 days, indistinct chlorotic local lesions were produced on inoculated leaves of *C. quinoa* and *C. anaranticolor* followed by systemic vein-yellowing and chlorosis after 10 days. Leaf distortion was also present and the plant became stunted with a loss of apical dominance. Of the viruses reported to infect *Spiraea* sp., the symptoms most resembled those attributed to ArMV. This virus was confirmed in gel diffusion tests using undiluted *C. quinoa* as antigen source and ArMV antiserum to an isolate from *Daphne*. Soil bait tests (as described in Chapter 3.18) were negative. The plants, therefore, were probably infected at the nursery.

Plate 48 Vein-yellowing and chlorotic mottling on
Spiraea bumalda "Anthony Waterer"
naturally infected with ArMV.



PART C : SENECIO GREYI

During the survey for virus symptoms on *Senecio greyi*, grown in flower beds at the University of Bath, two different virus-like symptoms were noticed. The leaves of one plant showed distinct vein-clearing and vein-yellowing symptoms (plate 49) especially on younger leaves. In another plant a translaminal "oak-leaf" like pattern symptom was observed on older leaves (plate 50).

Attempts were made to isolate virus from these two bushes using 0.05 M phosphate buffer pH 7.8 containing 7.5% PVP (insoluble) but were unsuccessful. A single dsRNA extraction from *Senecio* leaves with line pattern was also negative.

Plate 49 Vein-yellowing symptom observed on *Senecio greyi*. Healthy leaf on the left.



Plate 50 An "oak-leaf" line pattern symptom noticed on older leaves of *Senecio greyi*. Healthy leaf on the left.



CHAPTER 7 GENERAL DISCUSSION

Knowledge of viruses which infect woody or herbaceous food plants is considerable (Walkey, 1985; Tomlinson, 1987; Posnette and Cropley, 1963; Frazier et al., 1970). In contrast, with the notable exception of work done in Eastern Europe (Schmelzer, 1968; 1970), there have been few surveys on virus diseases of woody ornamentals. The most extensive work in the UK was probably that done by Sweet and colleagues in the 1970's (Sweet, 1976; Sweet and Campbell, 1976; Sweet and Sparks, 1977;). Worldwide literature on the subject was collated by Cooper (1979). More recently, Perkins (1987) conducted a limited survey on plants which were being assessed as part of the clonal selection scheme (CSS) for woody ornamentals produced in the UK (Webster 1988). Even though this material represented 'quality' clones sent in by the nurseries contributing to the CSS, about half of the plants tested were found to be infected (Perkins, 1987).

The survey done in the present study adopted a similar approach to Perkins (1987), although samples were collected from a wider range of sources - nurseries, parks, public gardens - as well as plants from the CSS.

Out of 30 species indexed by sap transmission to indicator plants eight were infected. This was similar to the results of an earlier survey (Perkins, 1987) in which eight out of 23 species tested were infected. Two genera (*Cassia*, *Caryopteris*) yielded more than one type of virus, and in one genus (*Cassia*) there was evidence for a mixed infection.

In 17 cases plants with symptoms did not contain a readily sap transmissible virus despite repeated attempts at isolation. One of these, *Fraxinus excelsior* L. had symptoms similar to those described by Cooper (1975 a), who found sap transmission of ArMV, the causal agent, to be infrequently successful. Problems in survey work can also arise from the often sporadic nature of symptom development in virus-infected woody ornamentals. Hicks (1979), for example, reported erratic symptom expression in rose cv "Golden Fleece" infected with *Prunus* necrotic ringspot virus. It was not uncommon to find symptom production followed by several years of apparent latency. In the present work, plants infected with ArMV (*Spiraea*) or CMV (*Caryopteris*, *Senecio*) showed similarly inconsistent symptom development. In tobacco plants infected by CMV, the recovery from

symptoms in systemically-infected leaves has been attributed to the presence of the satellite RNA (Kaper and Waterworth, 1981). In view of this, it would be interesting to know whether the apparent milder symptoms of the Norfolk *Senecio greyi* infected with CMV were associated with the presence of a satellite molecule. In one extraction from the more severely affected Bath plant, no satellite species was detected.

Inhibitors of virus infection are widely distributed in woody plants (Gyorgy, 1982), and may contribute to the failure of sap transmission (Matthews, 1981). In woody hosts (Fulton, 1966), these inhibitors are often assumed to be mainly phenolic compounds (tannins), which inactivate virus through the formation of bonds with carboxyl, thiol and amino groups on viral coat proteins. The virus particles may also be precipitated through the formation of cross-links between protein and polyphenols (or their quinones). Experiments in which virus and sap extracts were mixed before inoculation to test plants, indicated the presence of inhibitors in *Caryopteris clandonensis* and *Cassia corymbosa*. With *Cassia* extracts the inhibition was partially reversed by the use of PVP which probably acts as a peptide 'mimic' (Haslam et al ., 1989). The inhibition associated with

Caryopteris sap was partially overcome by inoculum dilution, a process known to reverse protein-polyphenol complex formation (Haslam et al., 1989). The presence of inhibitors, however, did not prevent isolation of two viruses from these hosts, presumably because of high levels of virus multiplication. Nevertheless, such substances, combined with low virus concentrations, may have contributed to the failure of transmission in some cases, during the survey.

The susceptibility of the test plant used may affect isolation (Matthews, 1981). In the present study, *Chenopodium quinoa* was the most consistently useful species in isolation attempts from woody ornamentals, a fact noted by Waterworth (1971) for viruses from fruit and ornamentals, as well as other workers on woody plants (Hicks, 1979; Perkins, 1987). Cucumber was not as useful a host as earlier work had indicated (Moore et al., 1948; Gendron and Kassanis, 1954). Cultivar susceptibility may play an important part; this was clearly shown for RRV from *Forsythia internedia* which infected "Parisienne Pickling" but not "Marketer".

The use of dsRNA analysis, was shown to be a feasible alternative to sap transmission for the detection of some viruses. However, in some

experiments, reported here, and by Hicks et al. (1988), dsRNA could not be detected in some woody ornamentals with symptoms. This may have been due to technical problems associated with dsRNA-cellulose binding in the presence of mucilaginous tissue extracts, or to low levels of replicative-form RNA in the leaves at sampling time.

The method, however, can provide diagnostic information for diseases of unknown aetiology, where no other technique will do so (Morris and Dodds, 1979). Thus dsRNA analysis should be considered as a "back-up" when infectivity assay fails, and where symptom expression indicates the presence of a virus-like agent. But, further work is needed to improve dsRNA yields from difficult hosts if the full diagnostic potential of the test is to be realised.

The viruses isolated in this study belonged to at least three taxonomic groups: the cucumoviruses (Kaper and Waterworth, 1981), the nepoviruses (Murant, 1981 a) and the closteroviruses (Lister and Bar-Joseph, 1981).

Cucumber mosaic virus was detected in two shrubs, *Caryopteris clandonensis* clone 7 from the CSS, and *Senecio greyi* from public gardens in

Norfolk and Bath. This virus has frequently been isolated from trees and shrubs in the UK (Cooper, 1979), while Schmelzer (1971) found it to be the most common virus infecting woody ornamentals in Eastern Europe. Cucumber mosaic virus isolates vary quite widely in their biological and physical properties (Francki and Hatta, 1980). The properties of the isolates from *Caryopteris* and *Senecio* came within the range expected for cucumoviruses (Francki et al., 1979; Kaper and Waterworth, 1981). The SV1 isolate, however, was slightly less stable than the others and in this respect resembled peanut stunt virus or tomato aspermy virus (Kaper and Waterworth, 1981).

Attempts to purify the isolate from *Caryopteris* were only partially successful, yields were low and the preparations not sufficiently pure to produce an antiserum. All three isolates, however, reacted in ELISA tests with an high titred antiserum to CMV from the American Type Culture Collection. The identity of the viruses from *Senecio* and *Caryopteris* could also be deduced from the dsRNA patterns.

Three nepoviruses were isolated from four hosts: SLRV from *Caryopteris clandonensis*, ArMV from *Cassia corymbosa* and *Spiraea*

bunalda, and RRV from *Forsythia intermedia* "Spectabilis". Harrison (1972) considered soil-borne viruses to be second only to aphid-transmitted viruses in importance, and the nepoviruses are probably the most prevalent group infecting hardy nursery stock in the UK (Sweet and Campbell, 1975a/b; Cooper and Sweet, 1976) and Eastern Europe (Schmelzer, 1971). Nepoviruses such as ArMV or SLRV can be spread locally by vector nematodes or by the dissemination of virus-infected propagating material. Ikin and Frost (1976) found widespread infection of rose cultivars with SLRV to be associated with the importation of symptomlessly infected *Rosa rugosa* rootstocks. In the present study, although the infection of *Caryopteris* with SLRV was associated with the presence of viruliferous nematodes, the infections of *Spiraea* or *Cassia* with ArMV were not. Of the nepoviruses isolated only SLRV was purified and an antiserum made, although both ArMV and RRV were purified in preliminary experiments.

The virus designated as CV1 from *Cassia corymbosa* was tentatively identified as a closterovirus largely on the basis of dsRNA analysis and the presence of several long filamentous particles (1000-2000 nm length) in leaf squashes.

Despite numerous attempts CV1 could not be purified, presumably because of the extreme fragility of the particles which is not uncommon within the closterovirus group (Lister and Bar-Joseph, 1981). The wide variation in particle length in leaf squashes would support this. Further work is needed on this virus to confirm its closterovirus affinities.

With many woody plant viruses, the woody host is not only a relatively poor source of inoculum, but is also difficult to infect by mechanical inoculation. This often makes it difficult to demonstrate a causal relationship between a virus and disease. Thus, Hicks (1979) failed to infect *Rosa* 'laxa' seedlings inoculated with purified preparations or crude sap extracts of *Prunus* necrotic ringspot virus. Young seedlings of several hosts, however, were susceptible enough to be infected mechanically by several viruses (Fulton, 1958). In the present study, single lesion cultures of SLRV or CV1 (closterovirus) were transmitted, respectively, to seedlings of *Caryopteris clandonensis* or *Cassia corymbosa*. No 'special' treatments were given before inoculation other than those normally used during routine sap transmission. Even so the infection rates were high considering the few plants available for

inoculation. Furthermore, there was little delay in symptom development usually found with return inoculations to woody hosts (Fulton, 1966); in *Cassia* first symptoms appeared a few weeks after inoculation.

Attempts to infect *C. corymbosa* seedlings with ArMV, or *C. clandonensis* seedlings with CMV were apparently not successful, although it was possible there was a long latent period before the virus reached detectable levels. This is especially likely for viruses that infect plants via the roots (Cadman, 1963) which may multiply preferentially in roots and in these cases root inoculation may be more successful. A new approach to satisfying Koch's second and third postulates was suggested by the work of Bellangher et al. (1988) who were able to return tobacco necrosis virus to aspen using tissue cultured plantlets. According to Nienhaus and Castello (1989) leaf tissue of such plantlets is more succulent, possesses a thinner cuticle, and a lower phenolic content than seedling tissue.

If a species or cultivar is widely infected with one or more virus, then the commercial benefits of establishing a 'nuclear stock' of virus-free material can be considerable. This is one of

the principles underlying the CSS for woody ornamentals in the UK (Webster, 1988). If a cultivar with desirable characters is infected with virus then application of techniques to eradicate infection should be considered (Walkey, 1985). These include meristem-tip culture, and chemotherapy or thermotherapy which are sometimes used in combination with tissue culture. In the present study, heat treatment of in vitro grown explants of *C. clandonensis* infected with SLRV, or incorporation of antiviral chemicals into the tissue culture medium, resulted in the elimination of virus, as judged by negative infectivity and ELISA assays. Both treatments induced some adverse effects on growth, particularly ribavirin which at high concentrations caused stunting of plants. Further work on virus elimination should include meristem-tip culture which has been used successfully for the elimination of virus from *Buddleia* (Duron and Morand, 1978) and *Daphne* (Sweet et al., 1979).

No previous reports on the elimination of SLRV from tissue cultured material was found. Testing the virus status of tissue cultured material directly after treatment, as done here, could save costs by avoiding the unnecessary culturing of plants which will later show infection.

However, there is the risk that the treatment has merely suppressed virus multiplication to levels below the sensitivity of the assay methods.

If tissue culture techniques are to be used successfully in virus eradication programmes, a suitable, rapid, micropropagation scheme for the woody ornamental will need to be worked out. Thus, attempts to initiate or proliferate cultures from *Senecio* or *Cassia* were unsuccessful and prevented further investigation of these species. Even with *Caryopteris*, rooting in vivo was not accomplished. If this remained unresolved, then 'micrografting' of virus-free shoot tips could be considered (Boxus and Druart, 1985).

Control measures, to be successful, require a deep understanding of virus ecology and spread. Viruses such as the nepoviruses, found in this study, are 'generalists' that have a wide host range, and survive in ecosystems with great species diversity (Thresh, 1983 a/b; Plumb and Thresh, 1983). That nepoviruses are common in woody plants probably reflects the preference of the vectors for stable habitats provided by long-lived perennials (Thresh, 1980). Thus, of the three nepoviruses recorded in the present work, ArMV was found in 19, and SLRV in nine of the 86 woody ornamental genera

described by Cooper (1979). In contrast, RRV was reported from two genera only, possibly because woody perennials form a much smaller part of the host spectrum of *Longidorus* spp. which transmit RRV, than *Xiphinena* spp. which transmit ArMV and SLRV (Murant, 1981).

None of the shrubs from which the nepoviruses were isolated -*Caryopteris*, *Spiraea*, *Forsythia* and *Cassia*- are normally grown as 'scions' grafted or budded onto vegetatively propagated or seedling rootstocks. Some of the shrubs (e.g. *Cassia*) however, can be propagated from seed. The occasionally high levels of seed transmission in nepovirus hosts may, therefore, represent a source of infection, particularly as such infections are often symptomless (Murant, 1981). Seed-borne infection of weed seed probably constitutes a more important natural reservoir, however, given the wide host range of the viruses and vectors (Harrison, 1977). The relationship of vector transmission to field outbreaks of disease, however, is not always clear. For example, Ikin and Frost (1976) recorded no infection within one year, among rose seedlings planted in soil containing viruliferous *X. diversicaudatum*. Similarly Sweet (1975 b) reported a low incidence of ArMV infection on ten nurseries growing a range of rosaceous plants. The importance

of vegetative propagation to the spread of nepoviruses is well illustrated by Cooper (1975). This author reported that most garden stocks of privet (over 90% in Oxford) were infected with ArMV but relatively few (about 10%) occurred in soils infested with virus-carrying nematodes.

Some larger nurseries and private companies are using tissue culture techniques to bulk up clonal material. If stock mother plants were infected then micropropagation could be a more efficient means of perpetuating the virus than conventional methods of propagation.

Cucumber mosaic virus (CMV) was the most common virus in the compilation of Cooper (1979) with 24 out of 86 genera infected. Three confirmed isolates of CMV were obtained from two genera of woody ornamentals, in the present study. Typically, CMV is transmitted by highly mobile aphid vectors and both virus and vector are widely distributed in the environment (Kaper and Waterworth, 1981). The virus behaves as an 'opportunistic' and woody (or herbaceous) hosts, in nurseries or gardens, are probably infected from outside sources. As with nepovirus infection, however, it is not certain if the virus is transmitted easily to woody plants. If it were so

and given the widespread occurrence of CMV, it is surprising that infection of woody ornamentals is not more common. Possibly the 'tougher' leaves in woody hosts makes it easier to dislodge virus from the aphid's mouthparts. Attempts to transmit aphid-borne viruses to forest tree species using vectors were unsuccessful (Nienhaus and Castello, 1989). The use of infected propagating material may still be the most important means for dissemination of CMV in woody ornamental trees and shrubs.

Foliar applications of insecticides may help to reduce populations of aphid vectors but are of limited effectiveness in reducing spread of viruses, like CMV, which are picked up and transmitted before the chemical has killed the aphid (Matthews, 1981). Experiments in Israel (Gera et al., 1979) have shown that mineral oil sprays can protect plants from infection by CMV.

According to Cooper (1979), rye is grown by some nurserymen in Britain as a packing material for their plants and might act as a barrier crop to reduce the rates of non-persistent virus transmission to low growing shrubs.

Woody ornamental nurseries are often ideal 'breeding grounds' for nematode vectors,

particularly *Xiphinema spp.* (Thomas, 1970), which have a wide host range among woody species. In addition, hedgerows surrounding nurseries may be hosts for both virus and vector (Harrison, 1977). Before planting new sites, therefore, it may be prudent to leave wide headlands, and to avoid planting recently cleared hedgerows for at least three years, unless the soil is partially sterilised (Cooper, 1979). Alternatively, these risks can be reduced by growing shrubs and trees in steam-sterilised horticultural soil-mixes. For nurseries where soil-borne infection is widespread, and where the economics of treatment are viable, there are several chemicals which will kill nematodes and reduce disease incidence (Harrison, 1977). However, while such treatments are successful for shallow-rooted crops, control can break down for trees or shrubs with large root volumes.

Data presented at the start of this thesis, together with other recent surveys (Cooper, 1979; Perkins, 1987) indicated that viruses and virus-like agents were widespread in woody ornamental genera. The establishment of a clonal selection scheme for woody ornamentals in the UK (Webster, 1988), however, will improve the health and quality of some of the more important genera and species. Nevertheless, the nurseryman can do much to

help himself by selecting good quality material for propagation.

More work is needed to assess the effects of viruses on the growth and propagation of woody ornamentals, and to establish the risks that trees and shrubs may pose as sources of viruses infecting plants cultivated for food.

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